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Third day of December 2004

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PROVISIONAL SPECIFICATION

Applicant(s):

THE UNIVERSITY OF WESTERN AUSTRALIA

Invention Title:

DETECTION SYSTEM AND USES THEREOF

The invention is described in the following statement:

DETECTION SYSTEM AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to a system for detecting molecular associations. In particular, the present invention relates to a multi-component detection system, wherein the molecular association of two or more components is detected.

BACKGROUND OF THE INVENTION

In the post genomic era proteomics has become more and more important. It includes the identification of all proteins encoded by the genome that are expressed in a cell, and the description of their behaviour, including expression, interactions and function.

Proteins do not act in isolation in a cell, but rather in stable or transitory complexes, with protein-protein interactions being key determinants of protein function (Auerbach *et al.* (2002) Proteomics, 2, 611-623). Furthermore, proteins and protein complexes interact with other cellular components like DNA, RNA and small molecules. Unravelling and dissecting out individual proteins involved in these interactions is crucial for the understanding of biological processes.

To this end a number of assay techniques have been developed over the years to assist in determining biological interactions. However, many of these techniques are either not suitable for high throughput screening or involve costly procedures. For example, techniques such as co-immunoprecipitation, have been used for many years to validate protein-protein interactions; however, this technique is not amenable to automation or high throughput screening. Other techniques such as co-

immunoprecipitation combined with mass spectrometry (Anderson & Mann (2000) FEBS Lett, 480, 25-31) is too complex, time consuming and expensive to be of use in drug screening programs. Surface plasmon resonance (SPR) is a highly sensitive and accurate technique capable of detecting biological interactions. However, this technique requires sufficient quantities of the purified target protein to be immobilised on the sensor surface and does not yield any information on the identity of ligands that may bind to it in a complex mixture of molecules.

Other techniques that have been developed include AlphaScreen system™, which is highly sensitive and versatile, but requires the interacting molecules to be available in a purified state; fluorescence polarisation and fluorescence anisotropy (Pope *et al.* (1999) Drug Disc Today, 4, 350-362), which is useful in high-throughput screening, but produces a number of false results and the dynamic range is limited; and fluorescence correlation spectroscopy (Pope *et al.* (1999) Drug Disc Today, 4, 350-362), which has a wide dynamic range, but the mass difference between the interacting partners must be large and the analysis is complex.

More importantly, all the above methods share the major disadvantage that the detection only occurs *in vitro*. This artificial situation does often not accurately reflect the intracellular environment where proteins interact and 'cross-talk' with many different partners. Also, interactions depend on buffer conditions and interactions may be abolished or initiated by the choice of inappropriate conditions, thus increasing the number and likelihood of false positive and false negative results.

As a consequence, a number of detection systems have been developed to detect protein-protein interactions '*in vivo*'. For example, the yeast 2-hybrid system (Fields &

Song (1989), Nature, 340, 245-246) has been widely used. However, this technique is only capable of monitoring protein-protein interactions inside the nucleus of living yeast cells. Therefore, the important class of membrane proteins and post-translational modifications specific to mammalian cells cannot be analysed.

Fluorescence resonance energy transfer (FRET) is another detection system capable of detecting *in vivo* protein-protein reactions (Forster (1948), Ann. Phys. 2, 57-75). This technique became particularly attractive and applicable to assays in living cells when the green fluorescent protein (GFP) and its mutant variants with different spectral characteristics were cloned. This allowed the genetic attachment of GFP and its variants to any target protein by fusing the encoding DNA sequences (Heim *et al.* (1994), Proc. Natl. Acad. Sci. USA. 91, 12501-12504). Compared to the yeast 2-hybrid system, FRET has the advantage that the monitored interactions can occur anywhere inside the cell. FRET can be determined in any cell type (mammalian, yeast, bacterial etc.) or cell-free system. It can be detected by fluorescence spectroscopy, fluorescence microscopy and fluorescence activated cell sorting (FACS). However, as discussed below, FRET has one major drawback, it can only be used to detect a single interaction.

Bioluminescence resonance energy transfer (BRET) is another technique that has been developed to study *in vivo* protein-protein interactions/reactions (Xu *et al.* (1999), Proc. Natl. Acad. Sci. USA 96, 151-156; Eidne *et al.* (2002), Trends Endocrin. Metabol. 13, 415-421). Similar to FRET, this technology has the advantage that the detection occurs within living cells and is not restricted to a particular cellular compartment. Additionally, it overcomes several potential limitations of FRET: as the light is generated intrinsically by the luciferase, the

detection system does not need to discriminate between the comparably weak signal resulting from the resonance energy transfer and the strong excitation light source. Furthermore, photo bleaching of the fluorophores and autofluorescence of the cells is not observed.

However, a major limitation of BRET, like FRET, is that only single, one-to-one interactions can be detected. However, it is widely accepted that most proteins have many more than one potential binding partner. Others act in larger complexes of two or more, and the function of a particular protein can critically depend on the presence of other proteins in the complex. Thus, looking at a single interaction does not address aspects of multiple functionality, specificity and cross-reactivity of a particular protein.

Consequently, there is a need for a multi-component detection system, which is capable of detecting multiple protein-protein associations *in vitro* and *in vivo*. More importantly, there is a need for a system that can analyse multiple associations in parallel, thus increasing throughput and reducing time and costs and a system that can analyse proteins and other biologically relevant molecules as part of multi-component molecular associates.

SUMMARY OF THE INVENTION

Inventors have now developed a multi-component detection system, which is capable of overcoming or at least alleviating some of the problems identified in the prior art systems, while still being capable of detecting multiple interactions *in vitro* and *in vivo*.

Accordingly, in a first aspect there is provided a multi-component detection system comprising:

i). a first agent comprising a first

interacting group coupled directly or indirectly to a first detection tag, which tag emits light of a first wavelength upon activation by a substrate or excitation light which produces a first activated detection tag;

ii). a second agent comprising a second interacting group coupled directly or indirectly to a second detection tag, which tag can accept the energy from the first detection tag in i) when the first and second interacting groups are associated and an appropriate substrate or excitation light for the first detection tag in i) is present thereby producing a second activated detection tag that emits light of a second wavelength;

iii). a third agent comprising a third interacting group coupled directly or indirectly to a third detection tag that can accept the energy from the second activated detection tag in ii) when the second and third interacting groups are associated and an appropriate excitation light for the second detection tag in ii) is present and that can accept the energy from the second activated detection tag in ii) when the first, second and third interacting groups are associated and an appropriate substrate or excitation light for the first detection tag in i) is present to produce a third activated detection tag that emits light of a third wavelength, but said third detection tag is not substantially activated by the first activated detection tag in i) when the first and third interacting groups are associated;

iv). an appropriate substrate or excitation light source to activate the detection tags in i) and ii); and

v). a means of detecting said emitted light.

In a second aspect there is provided a multi-component detection system comprising:

i). a first agent comprising a first interacting group coupled directly or indirectly to a first detection tag, which tag emits light of a first

wavelength upon activation by a substrate or excitation light which produces a first activated detection tag;

ii). a second agent comprising a second interacting group coupled directly or indirectly to a second detection tag, which tag can accept the energy from the first detection tag in i) when the first and second interacting groups are associated and an appropriate substrate or excitation light for the first detection tag in i) is present thereby producing a second activated detection tag that emits light of a second wavelength;

iii). a third agent comprising a third interacting group coupled directly or indirectly to a third detection tag consisting of a non-fluorescent quencher molecule that can accept the energy from:

a). the first activated detection tag when the first and third interacting groups are associated; and/or

b). the second activated detection tag when the second and third interacting groups are associated and an appropriate substrate or excitation light for the first and/or second detection tag is present, whereby the light emission from the first and/or second activated detection tag is decreased;

iv). an appropriate substrate or excitation light source to activate the detection tags in i) and ii); and

v). a means of detecting said emitted light.

In a third aspect there is provided a multi-component detection system comprising:

i). a first agent comprising a first interacting group coupled directly or indirectly to a first detection tag, which tag emits light of a first wavelength upon activation by a substrate or excitation light which produces a first activated detection tag;

ii). a second agent comprising a second interacting group coupled directly or indirectly to a second detection tag, which tag emits light of a second

wavelength upon activation by a substrate or excitation light, which produces a second activated detection tag;

iii). a third agent comprising a third interacting group coupled directly or indirectly to a third detection tag, which tag can accept the energy from the first activated detection tag when the first and third interacting groups are associated and an appropriate substrate or excitation light for the first detection tag is present to produce a third activated detection tag that emits light of a third wavelength;

iv). a fourth agent comprising a fourth interacting group coupled directly or indirectly to a fourth detection tag, which tag can accept the energy from the second activated detection tag when the second and fourth interacting groups are associated and an appropriate substrate or excitation light for the second detection tag is present to produce a fourth activated detection tag that emits light of a fourth wavelength;

v). an appropriate substrate or excitation light source to activate the first and second detection tags, and

vi). a means of detecting said emitted light.

In a fourth aspect there is provided a multi-component detection system comprising:

i). a first agent comprising a first interacting group coupled directly or indirectly to a first detection tag, which tag emits light of a first wavelength upon activation by a substrate or excitation light, which produces a first activated detection tag;

ii). a second agent comprising a second interacting group coupled directly or indirectly to a second detection tag, which tag can accept the energy from the first detection tag when the first and second interacting groups are associated and an appropriate substrate or excitation light for the first detection tag is present thereby producing a second activated detection

tag that emits light of a second wavelength;

iii). a third agent comprising a third interacting group coupled directly or indirectly to a third detection tag that can accept the energy from the first activated detection tag when the first and third interacting groups are associated and an appropriate substrate or excitation light for the first detection tag is present to produce a third activated detection tag that emits light of a third wavelength;

iv). an appropriate substrate or excitation light source to activate the first detection tag, and

v). a means of detecting said emitted light.

In one embodiment, the interacting groups are capable of associating with one or more other interacting groups. These associations may be between identical interacting groups or between different interacting groups or combinations thereof.

Preferably, the interacting groups are selected from the group consisting of compounds, proteins, protein domains, protein loops, protein termini, peptides, hormones, lipids, carbohydrates, nucleic acids, oligonucleotides, pharmaceutical agents, pharmaceutical drug targets, antibodies, antigenic substances, viruses, bacteria, and cells or any associate or complex thereof.

When the interacting group is a nucleic acid molecule then any form of nucleic acid molecule may be used. For example, the nucleic acid molecule might include genomic deoxynucleic acid (DNA), recombinant DNA, complimentary DNA (cDNA), peptide nucleic acid (PNA), ribonucleic acid (RNA), RNA including hetero-nuclear RNA (hnRNA), transfer RNA (tRNA), small interfering RNA (siRNA), messenger RNA (mRNA), or ribosomal RNA (rRNA) and hybrid molecules thereof.

In one embodiment, external stimuli are applied to directly or indirectly modulate associations and/or conformations of interacting groups. Preferably, stimuli are reagents including any known molecule, organic or inorganic, proteinaceous or non-proteinaceous, ligand, antibody, enzyme, nucleic acid, carbohydrate, lipid, drug compound, agonist, antagonist, inverse agonist or compound or complex thereof or a change of conditions including temperature, ionic strength or pH.

Detection tags according to this invention may be any known molecule, organic or inorganic, proteinaceous or non-proteinaceous or complex thereof, capable of emitting light via a chemical reaction or absorbing light in the near UV to near infra-red range or capable of fluorescence or phosphorescence. Preferably, the detection tag is a bioluminescent protein, a fluorescent protein, a fluorescent moiety or a non-fluorescent quencher.

Preferably, the bioluminescent protein is selected from the group consisting of luciferase, galactosidase, lactamase, peroxidase or any protein capable of luminescence in the presence of a suitable substrate.

Preferably, the fluorescent protein selected from the group consisting of green fluorescent protein (GFP) or variants thereof, blue fluorescent variant of GFP (BFP), cyan fluorescent variant of GFP (CFP), yellow fluorescent variant of GFP (YFP), enhanced GFP (EGFP), enhanced CFP (ECFP), enhanced YFP (EYFP), GFPS65T, Emerald, Topaz, GFPuv, destabilised EGFP (dEGFP), destabilised ECFP (dECFP), destabilised EYFP (dEYFP), HcRed, t-HcRed, DsRed, DsRed2, t-dimer2, t-dimer2(12), mRFP1, pocilloporin, Renilla GFP, Monster GFP, paGFP, Kaede protein and kindling protein, Phycobiliproteins and Phycobiliprotein conjugates including B-Phycoerythrin, R-Phycoerythrin and Allophycocyanin or any other protein capable of

fluorescence.

The fluorescent moiety can be any known fluorescent moiety. Preferably, the fluorescent moiety is selected from the group consisting of Alexa Fluor dyes and derivatives, Bodipy dyes and derivatives, Cy dyes and derivatives, fluorescein and derivatives, dansyl, umbelliferone, fluorescent and luminescent microspheres, fluorescent nanocrystals, Marina Blue, Cascade Blue, Cascade Yellow, Pacific Blue, Oregon Green and derivatives, Tetramethylrhodamine and derivatives, Rhodamine and derivatives, Texas Red and derivatives, rare earth element chelates or any combination or derivative thereof or any other molecule with fluorescent properties.

In one embodiment, at least one of the detection tags is a non-fluorescent quencher. The non-fluorescent quencher can be any known non-fluorescent chromophore with the ability to absorb light and to quench fluorescence and/or luminescence. The non-fluorescent quencher can therefore be any known proteinaceous or non-proteinaceous molecule. Preferably, the non-fluorescent quencher is selected from the group consisting of dabcyl, non-fluorescent pocilloporins, QSY-7, QSY-9, QSY-21, QSY-35, BHQ-1, BHQ-2 and BHQ-3.

The detection tags and interacting groups are directly or indirectly coupled. Preferably, the direct or indirect coupling is any known covalent or non-covalent means of coupling two molecules. More preferably, the direct or indirect coupling of the interacting groups and detection tags is selected from the group consisting of chemical cross-linking, chemical modification of proteins, chemical modification of amino acids, chemical modification of nucleic acids, chemical modification of carbohydrates, chemical modification of lipids or any other organic or inorganic molecule, non-covalent interactions including

biotin-avidin, antigen-antibody or nucleic acid hybridisation.

In one preferred embodiment, the interacting group and detection tag are part of the same polypeptide chain. For example, a nucleic acid molecule coding for a proteinaceous interacting group and a proteinaceous detection tag are optionally fused to:

(i) a sequence coding for a peptide sequence used for affinity purification of a fusion construct; and/or

(ii) a sequence coding for a peptide sequence which directs the fusion construct to a subcellular compartment of a eukaryotic cell; and/or

(iii) a sequence coding for a peptide sequence which facilitates the penetration of a eukaryotic cell membrane to produce a fusion protein of the interacting group, detection tag and said peptide(s).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows variations of multiplex interaction assays: (a) 'OR' assay, (b) 'parallel' assay. Abbreviations: DT-IG, detection tag attached to an interacting group; RET, resonance energy transfer.

Figure 2 shows aspects of assays for the analysis of multi-component molecular associates. Assays may monitor complete formation or dissociation of an associate (a) or a partial association/dissociation (b, c) when subsets of interactions can still be detected. DT1 and DT2 are activated while the signals are detected. Abbreviations: DT-IG, detection tag coupled directly or indirectly to an interacting group; RET, resonance energy transfer.

Figure 3 shows aspects of assays for the analysis of multi-component molecular associates using a non-

fluorescent quencher as DT3. Assays may monitor complete formation or dissociation of an associate or a partial association/dissociation when subsets of interactions can still be detected. DT1 and DT2 are activated while the signals are detected. Abbreviations: DT-IG, detection tag attached to an interacting group; RET, resonance energy transfer.

Figure 4 shows fusion protein constructs. Schematic representation of the multiple cloning sites of pETDuet-1 (Novagen). PCR products were cloned in-frame into 4 different sites. Oligonucleotide linkers encoding for a 12- or 18-aminoacid spacer could be inserted between subunits 1 and 2. The open reading frame encoded by the multiple cloning site of the vector provided a 15-aminoacid spacer between subunits 1 and 3 and a 7-aminoacid spacer between subunits 2 and 3. Abbreviations: T7 prom., T7 promoter sequence; T7 stop, T7 terminator sequence; His(6), histidine tag consisting of 6 consecutive histidine residues.

Figure 5 shows spectral properties of proteinaceous DTs. Fluorescence spectra of ECFP, EGFP and mRFP1 (a) show a large spectral overlap between ECFP and EGFP but only little overlap between ECFP and mRFP1. There is significant spectral overlap between ECFP and EYFP and also EYFP and mRFP1 (b). The ECFP emission overlaps surprisingly well with the t-dimer2(12) excitation (c).

Figure 6 shows FRET between proteinaceous DTs. The mRFP1-12-EGFP fusion protein shows a 5 times stronger fluorescence emission at 610 nm as a result of FRET than mRFP1 on its own which is not significantly excited at 480 nm. Spectra were normalised to the mRFP1 fluorescence when excited at 560 nm.

Figure 7 shows RET between Renilla luciferase (Rluc), a

bioluminescent protein and proteinaceous DTs: (a) no DT; (b) EGFP; (c) EYFP; (d) t-dimer2(12) and (e) mRFP1. Spectra were normalised to the emission maxima.

Figure 8 shows calculation of RET ratios for various fusion proteins. Shown are the values for the EGFP channel (a, c), the EYFP channel (b, e), the t-dimer2(12) channel (d, f) and the mRFP1 channel (g). Good separation is achieved between EGFP-t-dimer2(12) and EYFP-t-dimer2(12), whereas EGFP-EYFP and t-dimer2(12)-mRFP1 are too close for an independent, simultaneous detection. Although mRFP1 is separated well from EGFP and EYFP it is not substantially activated by Rluc resulting in only a small RET signal.

Figure 9 shows analysis of RET with non-proteinaceous DTs. Biotinylated Rluc was mixed with various streptavidin conjugates. Luminescence spectra are shown in black, fluorescence emission and excitation spectra of the conjugated dyes are shown in grey solid and dashed lines, respectively. The following conjugates were used: (a) Alexa Fluor 488, (b) Oregon green, (c) Alexa Fluor 555, (d) Alexa Fluor 568 and (e) Alexa Fluor 594. As a negative control non-biotinylated Rluc was used (f) which does not result in a RET signal.

Figure 10 shows linearity of assays using non-proteinaceous DTs. Serial dilutions of streptavidin-Oregon green (a) and streptavidin-Alexa Fluor 594 (b) mixed with biotinylated Rluc show a linear relationship between concentration and RET ratio when plotted on a log-scale.

Figure 11 shows multiplex RET detection. Mixtures of EGFP-15-Rluc/t-dimer2(12)-15-Rluc (a) and streptavidin-Oregon green/streptavidin-Alexa Fluor 594 (b) were analysed. In both models the 2 channels can be analysed simultaneously and quantitated independently.

Figure 12 shows RET in a complex molecular associate containing the proteinaceous DTs ECFP, EGFP and mRFP1. The mRFP1 fluorescence emission is increased in the presence of EGFP and is maximal at the excitation maximum of ECFP between 420 and 430 nm (a). FRET occurs between ECFP and EGFP when ECFP is excited between 420 and 430 nm (b). When EGFP is excited mRFP1 is activated (c).

Figure 13 shows analysis of the mRFP1-12-EGFP-Rluc construct. The luminescence spectrum (a) shows the activation of EGFP and mRFP1 when Rluc is activated by the presence of the substrate. The increase of the red fluorescence is significantly higher compared to the mRFP1-15-Rluc construct. When EGFP is activated by excitation light FRET occurs and mRFP1 is activated (b).

DETAILED DESCRIPTION OF THE INVENTION

Before describing the present invention in detail, it is to be understood that this invention is not limited to particularly exemplified bioluminescent or fluorescent proteins, analytes, or methods and may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting which will be limited only by the appended claims.

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety. However, publications mentioned herein are cited for the purpose of describing and disclosing the protocols, reagents and vectors which are reported in the publications and which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior

invention.

Furthermore, the practice of the present invention employs, unless otherwise indicated, conventional molecular biology, chemistry and fluorescence techniques, within the skill of the art. Such techniques are well known to the skilled worker, and are explained fully in the literature. See, eg., Coligan, Dunn, Ploegh, Speicher and Wingfield "Current protocols in Protein Science" (1999) Volume I and II (John Wiley & Sons Inc.); and Bailey, J.E. and Ollis, D.F., Biochemical Engineering Fundamentals, McGraw-Hill Book Company, NY, 1986; Lakowicz, J. R. Principles of Fluorescence Spectroscopy, New York : Plenum Press (1983) for fluorescence techniques.

It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a protein" includes a plurality of such proteins, and a reference to "an analyte" is a reference to one or more analytes, and so forth. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any materials and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred materials and methods are now described.

The present invention relates to a system for detecting multiple molecular associations. The term "molecular association" or "association" as used herein refers to a combination of two or more interacting groups associated via any known direct or indirect stabilising atomic or molecular level interaction or any combination thereof, where the interactions include, without limitation,

bonding interactions such as covalent bonding, ionic bonding, hydrogen bonding, coordinate bonding, or any other molecular bonding interaction, electrostatic interactions, a polar or hydrophobic interactions, or any other classical or quantum mechanical stabilising atomic or molecular interaction.

In one embodiment, the molecular association is between one or more agents comprising one or more interacting groups (IGs), wherein the IGs are coupled directly or indirectly to one or more detection tags. The term "agent" or "IG-DT agent" as used herein refers to a complex between an IG and a DT, ie. an IG coupled directly or indirectly to a DT. Agents may be engineered or modified to contain chemical groups, peptide sequences, proteins or nucleic acid molecules that may (i) facilitate their purification and/or (ii) target them to a subcellular compartment of a eukaryotic host cell and/or (iii) enable them to penetrate the cell membrane of a eukaryotic cell when added to the medium surrounding the cell.

Accordingly, the term "association" also refers to any interaction or conformational change involving interacting groups that brings the coupled detection tags into proximity. The distance between the detection tags is preferably in the range of between 1 and 10 nm. A direct physical contact between the IG-DT agents is not required and may be mediated by a third molecule and/or a third interacting group.

The term "interacting group" or "IG" as used herein encompasses compounds, proteins, protein domains, protein loops, protein-termini, peptides, hormones, protein-lipid complexes, lipids, carbohydrates, carbohydrate-containing compounds, nucleic acids, oligonucleotides, pharmaceutical agents, pharmaceutical drug targets, antibodies, antigenic substances, viruses, bacteria, and cells or any complex

thereof. Essentially, the interacting group is an entity capable of forming a complex with one or more entities. For example, an antibody in context with the present invention would be a first IG in that it is capable of forming a complex with an antigen, wherein the antigen would be the second IG (see *infra*). Another example, of an IG of the present invention would be a ligand, which is capable of forming a complex with a receptor. A further example is the interaction of an enzyme with its substrate. Additionally, the IGs may be part of the same molecule. Accordingly, for example, the third intracellular loop of a G-protein coupled receptor would be a first IG and the C-terminus of the same receptor would be a second IG which would associate when the receptor is activated or inactivated.

In one embodiment, external stimuli are applied to directly or indirectly modulate associations and/or conformations of interacting groups. The term "stimuli" as used herein refers to reagents including any known molecule, organic or inorganic, proteinaceous or non-proteinaceous, ligand, antibody, enzyme, drug compound, agonist, antagonist, inverse agonist, compound or complex thereof. It further refers to a change of external conditions including temperature, ionic strength or pH. Stimuli can act directly or indirectly. For example if stimuli are reagents they may physically bind to interacting groups and consequently mediate or prevent their association. This for example, could be a ligand that results in the dimerisation of a receptor or a conformational change within a receptor. An example for stimuli acting indirectly would be a reagent or change of conditions that activates an intracellular signalling pathway with the result that IGs are modified by cellular enzymes, for example phosphorylated; the modification in turn changes the associations of the IGs.

The term "detection tag" as used herein encompasses bioluminescent proteins, fluorescent proteins, fluorescent moieties and non-fluorescent quenchers. In short any known molecule, organic or inorganic, proteinaceous or non-proteinaceous or complex thereof, capable of emitting light via a chemical reaction or absorbing light in the near UV to near infra-red range or capable of fluorescence or phosphorescence.

The term "bioluminescent protein" as used herein refers to any protein capable of acting on a suitable substrate to generate luminescence. Bioluminescent proteins include luciferases, which have been found in bacteria, fungi, insects and marine creatures. They catalyse the oxidation of a specific substrate (generally known as luciferins) under light emission (Hastings (1996) Gene 173, 5-11). The most widely known substrate is coelenterazine which occurs in cnidarians, copepods, chaetgnaths, ctenophores, decapod shrimps, mysid shrimps, radiolarians and some fish taxa (Greer & Szalay (2002) Luminescence 17, 43-74). Two of the most widely used luciferases are:

(i) Renilla luciferase (*from R. reniformis*), a 35 kDa protein, which uses coelenterazine as a substrate and emits light at 480 nm (Lorenz *et al.* (1991) Proc. Natl. Acad. Sci. USA 88, 4438-4442); and

(ii) Firefly luciferase (from *Photinus pyralis*), a 61 kDa protein, which uses luciferin as a substrate and emits light at 560 nm (de Wet *et al.* (1987) Mol. Cell. Biol. 2987, 725-737).

More recently, Gaussia luciferase (from Gaussia princeps) has been used in biochemical assays (Verhaegen *et al.* Anal. Chem. 74: 4378-4385, 2002). Gaussia luciferase is a 20 kDa protein that oxidises coelenterazine in a rapid reaction resulting in a bright light emission at 470 nm.

In one embodiment, the bioluminescent proteins used with

the present invention exhibit an intense and constant light emission as long as the substrate is present. As the bioluminescent proteins are coupled to IGs, it is preferable to use bioluminescent proteins with a small molecular weight to prevent an inhibition of the interaction between the IGs due to steric hindrance. The bioluminescent proteins preferably consist of a single polypeptide chain to facilitate an easy production of the IG-DT agent. Also the bioluminescent proteins preferably do not form oligomers or aggregates, which could otherwise inhibit the function of the coupled IG. The bioluminescent proteins Renilla luciferase, Gaussia luciferase and Firefly luciferase meet all or most of these criteria.

The term "substrate" as used herein refers to any molecule that can be used in conjunction with a bioluminescent protein to generate luminescence in a chemical reaction.

The choice of the substrate can impact on the wavelength and the intensity of the light generated by the bioluminescent protein. For Renilla luciferase for example, coelenterazine analogues are available that result in light emission between 418 and 512 nm (Inouye *et al.* (1997) Biochem. J. 233, 349-353). A coelenterazine analogue (400A, 'DeepBlueC') has been described emitting light at 400 nm with Renilla luciferase (PCT application WO01/46691).

Substrates used with this invention are preferably cell-permeable and are able to pass the cellular membrane to become available to an intracellular bioluminescent protein. Coelenterazine and most of its derivatives are highly cell permeable (Shimomura *et al.* (1997) Biochem. J. 326: 297-298), whereas luciferin does not efficiently cross the membrane of mammalian cells. However, a caged luciferin compound has been developed that passes the cell membrane and is released by cellular enzymes or UV light

once inside the cytoplasm (Yang *et al.* (1993) Biotechniques 15, 848-850).

The term "fluorescent protein" as used herein refers to any protein capable of fluorescence or phosphorescence. There are a number of different fluorescent proteins that can be employed in this invention. For example, the most widely used fluorescent protein in molecular and cell biology are the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Tsien (1998) Annu. Rev. Biochem. 67, 509-544) and the variants derived from its sequence. 'Enhanced' fluorescent proteins (eg. EGFP) were developed by point mutations that increase the solubility and fluorescence and accelerate protein folding (Zernicka-Goetz *et al.* (1997) Development 124, 1133-1137). A Phe to Leu point mutation at position 64 has increased stability of the protein at 37°C and a Ser to Thr mutation at position 65 resulting in an increased fluorescence (Okabe, M. *et al.* (1997) FEBS Letters 407, 313-319; Clontech Palo Alto, Calif.). The EGFP, which is commercially available from Clontech, incorporates a humanised codon usage rendering it "less foreign" to mammalian transcriptional machinery and ensuring maximal gene expression. Additionally, the spectral properties of the green fluorescent protein can be altered by site-directed mutagenesis of specific amino acids, for example blue (EBFP), cyan (ECFP) and yellow (EYFP) mutants of EGFP have been produced (Zhang *et al.* (2002) Nat. Rev. Mol. Cell Biol. 3, 906-918). Another important class of fluorescent proteins is the red fluorescent proteins (RFP) from the coral species Discosoma (DsRed) (Matz *et al.* (1999) Nature Biotechnol. 17, 969-973) and *Heteractis crispa* (HcRed) (Gurskaya *et al.* (2001) FEBS Lett. 507, 16-20).

Preferably fluorescent proteins with a high fluorescence quantum yield are used with the present invention.

Preferably, the molecular weight of fluorescent proteins used with the present invention should be small enough to avoid steric hindrance between the IGs.

Preferably, monomeric proteins are used to avoid aggregation and interference with the function of a coupled IG. GFP forms a weak dimer but its tendency to dimerise can be minimised by the mutation of hydrophobic amino acids in the dimerisation interface (Zacharias *et al.* (2002) Science 296, 913-916). The red fluorescent protein DsRed is an obligate tetrameric protein. Recently, 17 point mutations of the DsRed sequence have been described that render DsRed into a dimeric protein (dimer2). The subunits of the dimer can be connected via a peptide linker to form a tethered dimer (t-dimer2(12)) that physically acts as a monomer. Additional 16 point mutations convert the dimer2 into a monomeric variant (mRFP1) (Campbell *et al.* (2002) Proc. Natl. Acad. Sci. USA 99, 7877-7882). The red fluorescent protein HcRed is a dimeric protein and is not fluorescent as a monomer. However, the two subunits can be fused by a short peptide linker connecting the C-terminus of the first subunit with the N-terminus of the second. This fusion protein (t-HcRed) acts effectively as a monomeric unit, similar to t-dimer2(12) (Fradkov *et al.* (2002) Biochem. J. 368, 17-21).

Preferably, fluorescent proteins used with the present invention exhibit short maturation times for the formation of their fluorophores. The fluorophore in these molecules is formed by specific re-arrangements of the polypeptide chain. This process can take from less than 1 h to more than 24 h (Zhang *et al.* (2002) Nat. Rev. Mol. Cell Biol. 3, 906-918). As a slow maturation process limits the availability and concentration of functional DT, the use of rapidly maturing proteins is preferred. Rapidly maturing fluorescent proteins are for example the green fluorescent protein EGFP and its colour variants and the

red fluorescent proteins t-dimer2 and mRFP1. Slow maturing proteins are for example DsRed and HcRed.

The terms "fluorescent moiety" or "fluorescent moieties" are used herein interchangeably and refer to non-proteinaceous molecules that are capable of generating fluorescence. Non-proteinaceous fluorescent molecules are usually small molecules that can be attached to other molecules. Each non-proteinaceous fluorescent molecule has specific spectral characteristics. There are a number of different fluorescent moieties that can be employed in this invention. Non-limiting examples include rhodamine, rhodamine derivatives, dansyl, umbelliferone, fluorescein, fluorescein derivatives, Oregon green, Texas Red, Alexa Fluor dyes and Cy dyes. A very attractive class of fluorescent moiety with regards to this invention are fluorescent nanocrystals (Bruchez *et al.* (1998) Science 281, 2013-2016). Fluorescent nanocrystals exhibit a strong fluorescence and their fluorescence emission can be adjusted by the crystal size over a wavelength range of more than 1000 nm. The excitation of all nanocrystals occurs at the same wavelength independent of their fluorescence emission. Therefore, various nanocrystals can be excited by the same light source or via RET from the same bioluminescent protein or fluorescent molecule.

Preferably fluorescent moieties with high fluorescence quantum yields are used.

A new type of fluorescent moiety was reported recently and involves both proteinaceous and non-proteinaceous components (Griffin *et al.* (1998) Science 281, 269-272; Adams *et al.* (2002) J. Am. Chem. Soc. 124, 6063-6076). The biarsenical-tetracysteine system fuses a short tetracysteine containing peptide to a target protein. This peptide forms a stable, fluorescent complex with a cell-permeable, non-fluorigenic biarsenical dye. Depending on

the molecular structure of the dye different fluorophores are obtained.

The term "excitation light" as used herein refers to any light source capable of activating a specific fluorophore. Non-limiting examples include lasers, Hg-lamps or Xe-lamps. The light source further has a means of limiting the emitted light to a specific wavelength or a specific range of wavelengths. This can be, for example, a suitable filter mounted to a filter wheel or a filter slide, a monochromator or lasers that only produce light of a single wavelength.

The term "non-fluorescent quencher" refers to any known proteinaceous or non-proteinaceous molecule, which is capable of absorbing fluorescence light without emitting light itself. Non-limiting examples are dabcyl, QSY quenchers, BHQ quenchers and non-fluorescent pocilloporin pigment proteins.

With regards to this invention, the bioluminescent, fluorescent proteins or fluorescent moieties should have suitable spectral properties for resonance energy transfer (RET) as well as certain physical characteristics. Their light emission should preferably be intense and constant as long as the necessary substrate is present. As the bioluminescent proteins and/or fluorescent moieties can be coupled directly or indirectly to IGs, it is most desirable to use small bioluminescent and fluorescent proteins to prevent an inhibition of the interaction between the IGs due to steric hindrance.

The terms "coupled directly or indirectly" as used herein means that the detection tag is attached to the IG to form an agent which is capable of being analysed or detected. The preferred method of coupling is determined by the nature of the IGs and DTs.

The bioluminescent or fluorescent proteins may be coupled (eg., covalently bonded) to a suitable IG either directly or indirectly (eg., via a linker group). Means of coupling bioluminescent or fluorescent protein to an agent are well known in the art. An example of a direct method of coupling a proteinaceous IG and a proteinaceous DT is genetic fusion, wherein the genes encoding the IG and the bioluminescent or fluorescent protein are fused to produce a single polypeptide chain.

Another example of a direct coupling method is conjugation, wherein the coupling of the IG with the fluorophore uses enzymes such as ligases, hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases.

Fluorescent moieties and non-proteinaceous, non-fluorescent quenchers have the disadvantage that their attachment to proteinaceous IGs is more difficult and often cannot occur inside live cells, in contrast to proteinaceous fluorescent moieties that can be genetically fused to proteinaceous IGs. An example of direct coupling of non-proteinaceous fluorescent moieties and non-fluorescent quenchers to IGs involves moieties covalently linked to reactive groups, which are able to form a covalent bond with specific chemical groups of the IG. Examples are iodoacetamides and maleimides reacting with SH-groups of cysteine residues, and succinimidyl esters, carboxylic acids and sulfonyl chlorides reacting with NH^{3+}-groups of lysine residues (Ishii *et al.* (1986) Biophys. J. 50, 75-89; Staros *et al.* (1986) Anal. Biochem. 156, 220-222; Lefevre *et al.* (1996) Bioconjug. Chem. 7, 482-489).

Another known way to attach a fluorescent moiety or a non-fluorescent quencher to the IG typically involves grafting a fluorescent moiety onto the IG or by incorporating the

fluorescent moiety into the IG during its synthesis. It is important that the labelled IG retains the critical properties of the unlabelled IG such as selective binding to a receptor or nucleic acid, activation or inhibition of a particular enzyme, or ability to incorporate into a biological membrane. There are a wide variety of fluorescent moieties available, including for example, dipyrrometheneboron difluoride dyes, rhodamine, rhodamine derivatives, Texas Red, dansyl, umbelliferone, etc. For a review of various labelling or signal producing systems that may be used, see U.S. Pat. No. 4,391,904.

One example of an indirect method of coupling a fluorescent moiety or non-fluorescent quencher to an IG such as a protein or nucleic acid, involves the covalent bonding of the fluorescent moiety or non-fluorescent quencher to a protein such as avidin, which is capable of binding biotin, wherein the biotin is covalently bound to the IG such that the IG and the fluorescent moiety or non-fluorescent quencher are coupled indirectly together via the interaction between biotin and avidin.

Another example of an indirect method of coupling the IG and bioluminescent or fluorescent protein is via a linker group. A linker group can function as a spacer to distance the bioluminescent or fluorescent protein from the agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in

the catalogue of the Pierce Chemical Co., Rockford, Ill.), may be employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidised carbohydrate residues. There are numerous references describing such methodology, eg., U.S. Pat. No. 4,671,958.

In one embodiment a proteinaceous DT or a proteinaceous IG is produced recombinantly by inserting a DNA sequence that encodes a DT or IG into an expression vector by standard molecular biology techniques well known to those skilled in the art. The DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the DNA sequence encoding the first polypeptides. Similarly, stop codons required to end translation and transcription termination signals are only present 3' to the DNA sequence encoding the second polypeptide. The polypeptide of the fused DT and IG is expressed in an appropriate host. Any of a variety of expression vectors known to those of ordinary skill in the art may be employed to express recombinant polypeptides of this invention. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line, such as CHO cells.

In another embodiment a proteinaceous IG-DT agent is produced recombinantly as a fusion construct. A DNA sequence encoding a fusion protein of the present invention is constructed using known recombinant DNA techniques to assemble separate DNA sequences encoding the proteinaceous DT polypeptide and the IG polypeptide into

an appropriate expression vector. The 3' end of the first DNA sequence is ligated, with or without a peptide linker, to the 5' end of the second DNA sequence so that the reading frames of both sequences are in phase to permit mRNA translation of the two DNA sequences into a single fusion protein that retains the biological activity of both the DT and IG. The orientation of DT and the IG within the fusion construct may be swapped to increase its functionality or expression.

A peptide linker sequence may be employed to separate the bioluminescent protein and IG polypeptide by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the bioluminescent protein or IG; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes or decrease the solubility of the fusion protein. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea *et al.* (1985) Gene 40, 39-46; Murphy *et al.* (1986) Proc. Natl. Acad. Sci. USA 83, 8258-8262; U.S. Pat. Nos. 4,935,233 and 4,751,180. The linker sequence may be from 1 to about 50 amino acids in length. Peptide sequences are not required when the bioluminescent protein or IG have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the DNA sequence encoding the first polypeptides. Similarly, stop codons require to end translation and transcription termination signals are only present 3' to the DNA sequence encoding the second polypeptide.

In one embodiment the sequence encoding the recombinant polypeptide is further genetically fused to a sequence encoding a peptide that facilitates the purification of the fusion construct via affinity chromatography. Examples include histidine tags, maltose-binding protein tags, cellulose-binding protein tags, intein tags, S-tags and GST tags.

In another embodiment the sequence encoding the recombinant polypeptide is genetically fused to a sequence encoding a peptide that facilitates the targeting of the fusion construct to a specific subcellular compartment of a eukaryotic host cell or for secretion into the surrounding medium. Examples include nuclear localisation signals, mitochondrial import sequences, KDEL sequences to target the endoplasmatic reticulum and export signals.

In yet another embodiment the sequence encoding the recombinant polypeptide is genetically fused to a sequence encoding a peptide that facilitates the penetration of eukaryotic cell membranes and thus the uptake of the fusion construct into the cell (Schwartz *et al.* (2000) Curr. Opin. Mol. Ther. 2, 162-167). Examples include peptide sequences derived from the HIV Tat protein, Herpes simplex virus VP22 and Kaposi FGF-4.

As an alternative to recombinant methods, polypeptides and oligopeptides can be chemically synthesised. Such methods

typically include solid-state approaches, but can also utilise solution based chemistries and combinations or combinations of solid-state and solution approaches. Examples of solid-state methodologies for synthesising proteins are described by Merrifield (1964) J. Am. Chem. Soc. 85, 2149; and Houghton (1985) Proc. Natl. Acad. Sci., 82, 5132.

Once the IGs have been labelled with the detection tags as described above, they can then be reacted with one or more other IGs, which also have attached thereto one or more detection tags.

In one embodiment all IG-DT agents are proteinaceous and coupled by genetic fusion to express IG-DT fusion constructs in a suitable host cell. The activation and detection of the DTs as well as an association of the IGs occurs inside the living host cell, inside cellular organelles, inside its cell membrane or at its surface.

In another embodiment a subset of IG-DT agents is proteinaceous and coupled by genetic fusion to express IG-DT fusion constructs in a suitable host cell. Another subset of IG-DT agents, proteinaceous, non-proteinaceous or combinations thereof, is added to the host cell with the optional ability of penetrating the host cell membrane. The activation and detection of the DTs as well as an association of the IGs occurs inside the living host cell, inside cellular organelles, inside its cell membrane or at its surface.

In yet another embodiment the IG-DT agents, regardless of their nature and of the method of preparations, are provided in solutions that may also contain suitable buffer substances. The IG-DT agents may be part of a cell extract, a cell fraction or a synthesis mixture, or may be at least about 90% pure, more preferably at least about

95% pure and most preferably at least about 99% pure. Purification occurs according to standard procedures of the art, including ammonium sulphate precipitation, affinity columns, ion exchange and/or size exclusion and/or hydrophobic interaction chromatography, HPLC, FPLC, gel electrophoresis, capillary electrophoresis and the like (see, generally, R. Scopes, Protein Purification, Springer-Overflag, N.Y. (1982), Deutsche, Methods in Enzymology Vol. 182: Guide to Protein Purification., Academic Press, Inc. N.Y. (1990)).

The present invention involves combinations of pairs of DTs, capable of being a donor and/or acceptor molecule. Accordingly, the DTs that can be used according to the present invention can be selected based on the physical properties thereof, as is known in the art of resonance energy transfer (RET), the two being selected so that they together comprise the donor and acceptor molecules of a RET pair. If one of the DTs within a RET pair is a bioluminescent protein, the RET is known as bioluminescence RET (BRET). If both DTs forming a RET pair are fluorophores the resulting RET is known as fluorescence RET (FRET). Examples of known suitable donor and acceptor pairs include:

Renilla luciferase and yellow fluorescent protein;

Renilla luciferase and green fluorescent protein;

Cyan fluorescent protein and yellow fluorescent protein;

fluorescein and tetramethylrhodamine;

5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS) and fluorescein;

See generally R. Haugland, Handbook of Fluorescent Probes and Research Chemicals (Sixth Ed. 1995). One or both of the fluorophores can be a fluorescent protein such as green fluorescent protein, and it is particularly advantageous to employ a fluorescent protein as the

fluorophore when the test compound is a protein or peptide by preparing a fusion protein of the test compound and a fluorescent protein.

The present invention involves the detection of multiple RET signals in parallel and combinations of bioluminescent or fluorescent moieties with specific spectral characteristics must be chosen. General spectral requirements and examples for combinations of these bioluminescent or fluorescent moieties depending on different embodiments as well as examples of applications are described below:

(i) 'AND' assays. In one embodiment the emission spectrum of DT1 sufficiently overlaps with the excitation spectrum of DT2 but not DT3. The excitation spectrum of DT3 sufficiently overlaps with the emission spectrum of DT2 while the emission maxima of DT1, DT2 and DT3 are sufficiently distinct to allow their separate detection (Figure 2 and Table 1).

TABLE 1

Types of assays and expected signals upon activation of DTs by an appropriate substrate or excitation light. Numbers indicate an increased signal of this DT.

Assay Type	Detectable IG association	activation of	
		DT1 activates	DT2 activates
AND	none, 1:3	1	2
	1:2	2	2
	2:3	1	3
	1:2:3	3	3
OR	none, 2:3	1	N/A
	1:2	2	
	1:3	3	
parallel	none	1	2
	1:3	3	2
	2:4	1	4

FRET systems involving three fluorescent moieties, all coupled to a short single-stranded oligonucleotides were reported recently (Tong *et al.* (2001) J. Am. Chem. Soc. 123, 12923-12924; U.S. patent number 6,627,748; Haustein *et al.* (2003) Chemphyschem. 4, 745-748). Those DNA molecules act as probes with new fluorescent labels distinct from labels consisting only of single fluorescent moieties. FRET occurs from both the first and second fluorophore to the third fluorophore increasing the signal obtained from the third fluorophore. A similar system was applied to monitor conformational changes within a short double-stranded DNA molecule (Liu *et al.* (2002) J. Am. Chem. Soc. 124, 15208-15216). In the present invention however, the first DT does not substantially activate the third DT, and although the energy decreases with every RET it is still surprisingly efficient after two steps and results in an easily detectable activation of DT3. An example of a suitable combination of DTs is Renilla luciferase using a standard coelenterazine substrate (emitting at 460-480 nm) as DT1 in combination with EGFP or EYFP (DT2) and mRFP1 (DT3). Another example is ECFP or Renilla luciferase using DeepBlue^{C} (emitting at 400 nm) as DT1 in combination with ECFP (DT2) and the red fluorescent protein t-dimer2 (DT3). Alternatively, EGFP or EYFP as DT2 may be substituted by the biarsenical dye FlAsH or a fluorescent moiety with similar spectral properties. Examples include Alexa Fluor 488 and Oregon green 514. The red fluorescent proteins mRFP1 and t-dimer2 may be substituted by others fluorescent proteins or fluorescent moieties with similar spectral properties, for example Alexa Fluor 546 or Alexa Fluor 568.

(ii) 'AND quencher' assays. In another embodiment the emission spectrum of DT1 sufficiently overlaps with the excitation spectrum of DT2. DT3 is a non-fluorescent quencher with an absorbance spectrum that sufficiently overlaps with the emission spectra of DT1 and/or DT2, and

preferably with the emission spectra of both DT1 and DT2 so that the emission of DT1 and/or DT2 is quenched (ie. decreased) when DT3 is in proximity to DT1 and/or DT3 (Figure 3 and Table 2). This system may be extended to include a fourth or more IG-DT agents (Table 2). Examples of suitable DTs include ECFP as DT1, EYFP as DT2 and dabcyl as DT3. Alternatively, EYFP may be used as DT1, mRFP1 as DT2 and QSY-7 as DT3. DT1 and/or DT2 may be replaced by fluorescent moieties, which form suitable FRET pairs, for example Alexa Fluor 488 in combination with Alexa Fluor 555 and QSY-7. A bioluminescent protein may be used as DT1, for example Renilla luciferase emitting at 460-480 nm in combination with EYFP as DT2 and QSY-7 as DT3. Preferably the emission of the bioluminescent protein is stable for the duration of the detection so that changes in the emission can be attributed to quenching. A decrease of the emission due to a decreasing substrate concentration may be corrected by detecting a control reaction or by applying a mathematical function describing the decay of the DT1 emission.

One example for the application of this and the previous embodiment of the invention is the analysis of cytokine receptor signalling. Cytokine receptors form hetero-dimers of membrane-bound subunits when activated by binding of their ligand. One subunit is usually specific for the ligand whereas the other one is responsible for signal transduction and is shared by other ligand-specific subunits. The activated receptors interact with intracellular proteins like signal transducer and activator of transcription (STAT) proteins (Ishihara *et al.* (2002) Biochim. Biophys. Acta 1592, 281-296). Thus cytokine receptor signalling involves a network of signal transducing molecules and receptor molecules with many overlapping and redundant functions. It is often difficult to attribute a particular effect to the actions of

TABLE 2

Assays involving non-fluorescent quenchers as DTs and expected signals upon activation of DTs by an appropriate substrate or excitation light. Numbers indicate an increased signal of this DT, none indicates a decreased signal/no activation of other DTs.

assay	Detectable IG association	Activation of DT1 activates	DT2 activates	DT3 activates
AND quencher 3-way 1, DT3 is a quencher for DT2 only	none, 1:3	1	2	N/A
	1:2	2	2	
	2:3	1	none	
	1:2:3	none	none	
AND quencher 3-way 2, DT3 is a quencher for DT1 and DT2	none	1	2	N/A
	1:2	2	2	
	1:3	none	2	
	2:3	1	none	
	1:2:3	none	none	
AND quencher 4-way 1, DT1 activates DT2 but not DT3, DT4 is quencher for DT2 and DT3	none, 1:3, 1:4	1	2	3
		2	2	3
	1:2	1	3	3
	2:3	1	none	3
	2:4	3	3	3
	1:2:3	none	none	3
	1:2:4	1	2	none
	1:3:4, 3:4	1	none	none
	2:3:4	none	none	none
	1:2:3:4			
AND quencher 4-way 2, DT1 activates DT2 and DT3, DT4 is quencher for DT2 and DT3	none, 1:4	1	2	3
	1:2	2	2	3
	1:3	3	2	3
	2:3	1	3	3
	2:4	1	none	3
	3:4	1	2	none
	1:2:3	2, 3	3	3
	1:2:4	none	none	3
	1:3:4	none	2	none
	2:3:4	1	none	none
	1:2:3:4	none	none	none

specific molecules or receptors. IGs can be derived from receptor subunits forming a suitable RET pair (DT1-IG1:IG2-DT2) when the receptor is activated and dimerises.

To further monitor the activation of a particular signal transducer by the receptor, a third IG is derived from the signal transducing protein (IG3-DT3). When this molecule interacts with the activated receptor complex energy is transferred from DT2 to DT3 and light of a specific wavelength is emitted from DT3 or the signal from DT2 and/or DT1 is quenched. This signal is specific for the activation of this particular pathway by this particular cytokine receptor. Activation of the same pathway via another cytokine receptor or activation of another pathway by the same cytokine receptor does not give a signal.

Another example is the analysis of G-protein coupled receptors (GPCRs) that form homo or hetero-dimers. Recent studies have shown that GPCRs may not only act as monomers but also as homo- and hetero-dimers which causes altered ligand binding, signalling and endocytosis (Rios *et al.* (2000) Pharmacol. Ther. 92, 71-87). The effect of drugs acting as agonists or antagonists of a specific receptor may therefore depend on the binding partners of this receptor. It may be desirable to limit the effect of a drug to a cellular response mediated by a specific receptor dimer. The system provided by this invention monitors the activity of a specific GPCR dimer. The GPCRs themselves act as IGs and are attached to DTs (IG2-DT2, IG3-DT3). A third IG (IG1-DT1) is derived from a molecule that interacts with GPCRs upon ligand binding (eg. ß-arrestin). A signal obtained from DT3 or a decrease of the DT2 and/or DT1 signal(s) indicates that the GPCR dimer is activated, whereas a signal from only DT2 indicates an activation of the IG2 GPCR in its monomeric state or bound to a GPCR other than IG3.

Another example is the transcriptional regulation of gene expression. Transcription factors act in multiprotein-DNA complexes and the composition of these complexes determines their specificity and activity (Wolberger *et al.* (1999) Ann. Rev. Biophys. Biomol. Struct. 28, 29-56). For example the transcription factor *Fos* is only active as a hetero-dimer with a member of the Jun transcription factor family (Chinenov *et al.* (2001) Oncogene 20, 2438-2452). The *Fos/Jun* dimer can activate or repress the transcription of numerous genes. The specificity and activity of the complex is regulated by additional proteins interacting with the dimer, like ETS transcription factors, NF-AT or Smad proteins (Wang *et al.* (1994) Mol. Cell Biol. 14, 1153-1159; Stranick *et al.* (1994) J. Biol. Chem. 272, 16453-16465; Zhang *et al.* (1998) Nature 394, 909-913). IGs can be derived from *Fos* and *Jun* proteins attached to DTs forming a suitable RET pair (DT1-IG1:IG2-DT2). This RET signal indicates a functional dimer of a particular *Fos/Jun* combination. The third IG is derived from a transcriptional regulator interacting with the Fos/Jun complex. This IG is attached to a third DT (IG3-DT3) that emits or quenches light transferred from DT2 when IG3 interacts with the IG1:IG2 complex. This signal is specific for the activity of the trimeric complex involving a particular combination of *Fos/Jun* proteins. Activation of *Fos/Jun* by interaction with other regulators or activation of different *Fos/Jun* complexes with the same regulator will not be detected.

Another example is the development of novel antiviral drugs. A major problem of therapies for HIV and other viruses is the adaptability of the virus by point mutations of viral proteins to gradually become resistant to all drugs being developed so far. Therapies that target multiple events in the viral life cycle are therefore more successful, and mixtures of different drugs, so-called combination therapies have found wide clinical use. A

promising, novel group anti-retroviral drugs are virus entry inhibitors (Starr-Spires *et al.* (2002) Clin. Lab. Med. 22, 681-701). The entry of HIV virions is mediated via two cellular receptors: CD4 and CXCR4 or CCR5, depending on the virus strain. Antibodies or drugs only blocking the virus-CD4 interaction rapidly loose their efficiency as the viral surface changes. The system provided by this invention allows the simultaneous detection of the viral binding to both receptors. The two receptors plus the viral surface protein can be labelled with DTs yielding a specific signal when the trimeric complex is formed. Thus, compounds can be identified that efficiently block both interactions or inhibit required conformational changes of the viral protein to bind to both receptors. As two vital interactions are targeted simultaneously the emergence of resistant viruses is less likely.

In another example, the invention is used to analyse the composition, conformation, assembly or dissociation of a large, stable molecular complex. The presence or absence of the different RET signals indicates the assembly and functionality of the complex or conformational changes/movements of within the complex or components of the complex. Examples of complexes include transcription factor complexes, ribosomes, proteasomes, chaperones, oligomeric receptors, ion channels etc.

Generally, for high-throughput screening and drug discovery, this type of assay can be used to find compounds inhibiting or activating the function of a molecule in its environment within a specific multi-component molecular associate. The function of the same molecule within another associate may not be affected.

It is clear to those skilled in the art that the aspects of molecular interaction as described above play an

important role in numerous cellular functions and are not limited to those described in the examples.

(iii) 'Parallel' assays. In a further embodiment the emission spectrum of DT1 sufficiently overlaps with the excitation spectrum of DT3 and the emission spectrum of DT2 sufficiently overlaps with the excitation spectrum of DT4 while the emission maxima of DT1/DT2, DT3 and DT4 must be sufficiently distinct to allow their separate detection whereas the emission spectra of DT1 and DT2 may be identical (Figure 1 and Table 1). Examples of DT1/DT2 include Renilla luciferase or ECFP in combination with EGFP or EYFP (DT3) and DsRed and its variants dimer2 and t-dimer2(12)(DT4). Renilla luciferase, ECFP as DT1/DT2 and EGFP and DsRed as DT3 and DT4 may be substituted by other molecules with similar spectral properties. As an example for an embodiment where DT1 and DT2 are not identical, ECFP may be used as DT1 in combination with EGFP (DT3) and firefly luciferase or EYFP (DT2) may be used in combination with mRFP1 (DT4). The emission spectra of EYFP and firefly luciferase are not sufficiently separated from EGFP. However, a spectral resolution can be achieved by exciting ECFP and detecting the EGFP emission first and then exciting EYFP while detecting the mRFP1 emission. Alternatively, coelenterazine may be added first, followed by the addition of luciferin after the first reaction has ceased. ECFP, EYFP and mRFP1 may be substituted by other fluorescent proteins or fluorescent moieties with similar spectral properties.

One example of the application of this embodiment of the invention is the monitoring of two different parts of a cellular signal transduction cascade. Signals are relayed from the activated receptor to their effective intracellular site via a cascade of interacting and each other activating or deactivating proteins. A well-characterised example is the MABK/Erk pathway (Cobb *et al.*

(1999) Prog. Biophys. Mol. Biol. 71: 479-500; Lewis *et al.* (1998) Adv. Cancer Res. 74, 49-139). The MAPK/Erk signalling cascade is activated by a wide variety of receptors involved in growth and differentiation including receptor tyrosine kinases (RTKs), integrins, and ion channels. Pairs of IGs may be derived from different interacting pairs of signalling molecules of the cascade. Each interacting pair gives a specific RET signal (DT1-IG1:IG3-DT3 and DT2-IG2:IG4-DT4) indicating the activation of a specific step. This may allow the simultaneous monitoring of one step upstream in the cascade (eg. SOS-Ras) and another one further downstream (eg. MEK-Erk). This type of assay is useful to identify components of the cascade that lie between the two detected steps. In high-throughput screening and drug discovery it may be used for the identification of drugs manipulating molecules between the two detected steps.

In another example, the assay is used to distinguish between the function of a mutant versus a normal protein. The role of mutant activated receptor protein-tyrosine kinases (PTKs) in oncogenesis is well established. An important principle in the activation of receptor PTKs is ligand-mediated dimerisation. Increasing evidence indicates that oncogenic activation of receptor PTKs occurs through mutations that lead to constitutive dimerisation and activation of the cytoplasmic catalytic domain (Hunter *et al.* (1997) Cell 88, 333-346). One example is the Tel-PDGFb receptor fusion, generated by the t(5:12) translocation in chronic myelomonocytic leukaemia. The N-terminal part of Tel, an Ets family transcription factor, is joined with the entire cytoplasmic domain of the PDGFb receptor PTK gene, resulting in dimerisation and constitutive PTK activation (Golub *et al.* (1994) Cell 77, 307-316). The assay system provided in this invention derives IGs from the receptor components and monitors the activity of both the defective (mutant) and the wild-type

(normal) receptor PTK. Thus, in high-throughput screening and drug discovery compounds can be identified specifically targeting the mutant receptor without interfering with the normal receptor function. This allows the identification of highly specific compounds during the first primary screening step.

In yet another example this type of assay may be used to provide built-in controls for the compounds used in high-throughput screening. It is a common problem that compounds interfere with protein function in general rather than specifically with the function of the target protein resulting in a false positive signal. With the assay system provided by this invention the targeted interaction may be monitored by one RET pair (DT1-IG1:IG3-DT3). A second, related interaction may be monitored in parallel (DT2-IG2:IG4-DT4). Only compounds exhibiting an effect on the first pair but not on the second are target-specific. A compound with effects on both targets acts via an unspecific effect.

In yet another example this type of assay may be used to identify substances toxic for a particular organism but not another, ie. a substance killing a parasite but not the host. A vital protein-protein interaction may be monitored with IGs derived from the parasite's proteins (DT1-IG1:IG3-DT3). In parallel the equivalent interaction with IGs derived from the host organism is monitored (DT2-IG2:IG4-DT4). The assay allows the identification of substances that are able to discriminate between the parasite's and the host's proteins.

Generally, for high-throughput screening and drug discovery this type of assay may be used to increase the throughput as two different interactions and functions are screened at the same time. This results in significant savings in reagents, cost and time.

It is clear to those skilled in the art that the aspects of molecular interaction as described above play an important role in numerous cellular functions and are not limited to those described in the examples.

(iv) 'OR' assays. In yet another embodiment the emission spectrum of first acceptor fluorophore (DT1) sufficiently overlaps with the excitation spectra of both the second fluorophore (DT2) and subsequent fluorophores (DT3+) while the emission maxima of DT1, DT2 and DT3 are sufficiently distinct to allow their separate detection (Figure 1 and Table 1). An example of a suitable combination of DTs is Renilla luciferase (emitting at 460-490 nm) or ECFP to be used as DT1 in combination with EGFP or EYFP (DT2) and DsRed, dimer2 or t-dimer2(12) (DT3). Although DsRed and the dimeric variants absorb only weakly below 500 nm they form a surprisingly strong RET acceptor. Alternatively, the biarsenical dyes FlAsH and ReAsH may be used as DT2 and DT3 (Adams *et al.* (2002) J. Am. Chem. Soc. 124, 6063-6076). Another alternative for DT2 and DT3 are fluorescent moieties with similar spectral properties as EGFP or EYFP and DsRed. Examples include Alexa Fluor 488, Oregon green 514 and Alexa Fluor 546. Yet another alternative for DT2 and DT3 are fluorescent nanocrystals. All nanocrystals absorb light below 500 nm, independent of their emission wavelength (Bruchez *et al.* (1998) Science 281, 2013-2016), making them ideal RET acceptors for this type of assay.

One example of an application of this embodiment of the invention is the monitoring of signal transduction pathways. Most cellular signalling events involve networks of interacting proteins, relaying a signal from a receptor to a response, usually involving gene transcription in the nucleus. The IGs can be components of a signalling pathway with IG1 relaying a signal to IG2 and IG3 or IG1 acting as the signalling link between IG2 and IG3. Examples of

signalling molecules of which the IGs can be derived from are *ras* and *raf* proteins, protein kinase C, MEK proteins etc. (Dikic *et al.* (1999) Cell Biochem. Biophys. 30, 369-387; Gutkind *et al.* (1988) Oncogene 17, 1331-1342; Luttrell *et al.* (1999) Curr. Opin. Cell Biol. 11, 177-183; Rozengurt *et al.* (1998) J. Cell Physiol. 177, 507-517).

Another example is the transcriptional regulation of gene expression. Transcription factors act in multiprotein-DNA complexes and the composition of these complexes determines their specificity and activity (Wolberger *et al.* (1999) Ann. Rev. Biophys. Biomol. Struct. 28, 29-56). For example, the transcription factor *Fos* forms heterodimers with different members of the Jun transcription factor family, depending on the cellular differentiation, growth, external stimuli etc. (Chinenov *et al.* (2001) Oncogene 20, 2438-2452). IGs can be derived from *Fos* and *Jun* family members monitoring selectively the state and activity of these important transcriptional regulators.

Generally, for high-throughput screening, this type of assay can be used to find compounds specifically inhibiting one interaction (eg. IG1:IG2) but not the other (IG1:IG3). This is important as the first interaction may cause a different cellular effect than the second, only one of which may be the desired effect of a drug. Therefore, this type of assay facilitates the development of drugs highly specific for a cellular effect.

It is clear to those skilled in the art that the aspects of molecular interaction as described above play an important role in numerous cellular functions and are not limited to those described in the examples.

The term "detecting emitted light" as used herein refers to any detection device capable of detecting photons of a specific wavelength in a quantitative manner. Examples

include photomultiplier tubes or CCD cameras. The detector further comprises a means of restricting the detected light to a specific wavelength or a specific range of wavelengths. This can be for example suitable filters mounted to a filter wheel or a filter slide or a monochromator.

In one embodiment the first DT is activated by excitation light specific for this DT and the light emitted by this DT and the other DTs is detected. Then the second DT is activated and the emitted light of this and other DTs is detected and so forth. The combined information provided by these sequential readings provides information on the associations between the IGs as summarised in Tables 2 and 3. This sequence of activation and detection may be repeated in time intervals to obtain kinetic data. At any time of this detection sequence substances can be added or conditions may be changed that may influence the associations of the IGs.

In another embodiment a suitable substrate is added for the activation of a first DT. The emitted light of this DT and the other DTs is detected while the excitation light is turned off or blocked. Then, excitation light specific for the activation of a second DT may be turned on and the emitted light of this DT and the other DTs is detected. To obtain kinetic data the detection mode can be switched continually between luminescence and fluorescence detection with the light source turned off and on, respectively. At any time of this detection sequence substances can be added or conditions may be changed that may influence the associations of the IGs.

In yet another embodiment a substrate suitable for a first DT is added. The emitted light of this DT and the other DTs is detected while the excitation light is turned off or blocked. When this first substrate is used up and the

light emission from the first DT ceased, a second substrate suitable for a second DT is added. The emitted light of this DT and the other DTs is detected while the excitation light is turned off or blocked. At any time of this detection sequence substances can be added or conditions may be changed that may influence the associations of the IGs.

The invention will now be further described by way of reference only to the following non-limiting examples. It should be understood, however, that the examples following are illustrative only, and should not be taken in any way as a restriction on the generality of the invention described above. In particular, while the invention is described in detail in relation to the use of specific detection tags and interacting groups, it will be clearly understood that the findings herein are not limited to these tags or interacting groups.

EXAMPLE 1 ANALYSIS OF PROTEINACEOUS FRET PAIRS

For assays according to this invention it is important to have detection tags with sufficient spectral overlap to from a RET pair and others that are separated enough that no RET occurs. The efficiency of RET can be described by the Foerster radius R_0. R_0 is the distance at which energy transfer is 50% efficient, ie. 50% of excited donors are deactivated by FRET. The magnitude of R_0 is mostly dependent on the spectral properties of the donor and acceptor dyes:

$$R_0 = \left[8.8\times10^{23} * \kappa^2 * n^{-4} * QY_D * J(\lambda)\right]^{1/6} \quad \text{Å}$$

where $?^2$ = dipole orientation factor (range 0 to 4; 2/3 for random orientation)
QY_D = fluorescence quantum yield of donor in the absence of acceptor or luminescence capacity of

a bioluminescent protein
n = refractive index (1.33 for water, depends on temperature, ionic strength)
J(?) = spectral overlap integral

To adjust the efficiency of RET the selection of dyes with a high quantum yield and sufficient spectral overlap (ie. J(?) is large) is the most important variable. Assays according to this invention occur in an aqueous medium suitable for biological molecules, therefore there is little variation in the refractive index n. The geometric orientation of the dyes, ie. the dipole orientation factor $?^2$, will be near $^2/_3$ in most situations, the value for randomly orientated molecules. This is because the tagging occurs with flexible linkers and spacing groups that allow the dyes more or less free rotation relative to the attached interacting group, although the use of bulky fluorescent proteins as DTs may limit the rotational freedom.

Consequently, the spectral properties of existing fluorescent proteins and their use as DTs according to this invention were examined. As a simple model system for interactions to investigate potential RET, fusion proteins of proteinaceous DTs were generated. This is the most ideal, permanent interaction and therefore well suited to define the magnitude of which RET can occur. The DT subunits were separated by linkers which had lengths of 7-18 amino acids. The linker sequences contained mostly serine and glycine residues for maximum flexibility. This allowed free rotation of the subunits against each other and prevented a loss of the RET signal due to an unfavourable geometric orientation.

The coding sequences of mRFP1, t-dimer2(12), ECFP, EGFP and EYFP were amplified via PCR with the following oligonucleotides (Table 3): mRFP1-fw/re, template: pRSETB-

mRFP1 (Campbell et al. Proc. Natl. Acad. Sci. USA 99, 7877-7882, 2002); t-dimer2(12): mRFP1-fw/t-dimer2(12)-re, template: pRSETB-t-dimer2(12) (Campbell et al. Proc. Natl. Acad. Sci. USA 99, 7877-7882, 2002); EGFP-P3-fw/re, template: pECFP-N1 (Clontech); EGFP-P2-fw/re, template:

TABLE 3

Oligonucleotide Sequences

Oligo	Sequence
mRFP1-fw	GACGATGACGATAAGGATCCGATG
mRFP1-re	CTTCGAATTCGAGGCGCCGGT
t-dimer2(12)-re	TCAAGCTTCGAATTCGACAGGAAC
EGFP-P2-fw	TATAGAGCTCGGTGAGCAAGGGCGAGGAGCTG
EGFP-P2-re	ATATAGTCGACCTTGTACAGCTCGTCCATGCCG
linker-12-fw	AATTCTGGCAGCGGTTCCGGCTCTGGTAGCT
linker-12-re	ACCAGAGCCGGAACCGCTGCCAG
Rluc-P3-fw	TAAAATTGCGGCCGCTTCCAAGGTGTACGACCCCGA
Rluc-P3-re	TATACTTAAGTTACTGTTCGTTCTTCAGCACGC
EGFP-P1-fw	TAGGATCCGGTGAGCAAGGGCGAGGAGCTG
EGFP-P1-re	TAGAATTCCCCTTGTACAGCTCGTCCATGCCG
linker-18-fw	AATTCTGGCAGCGGTTCCGGCTCTGGTTCTGGCAGCGGTAGCGGTAGCT
linker-18-re	ACCGCTACCGCTGCCAGAACCAGAGCCGGAACCGCTGCCAG
DsRed2-P1-fw	TTGGATCCGGCCTCCTCCGAGAACGTCATCAC
DsRed2-P1-re	TAGAATTCGACAGGAACAGGTGGTGGCGGC
DsRed2-P2-fw	TATAGAGCTCTGCCTCCTCCGAGAACGTCATCAC
DsRed2-P2-re	TATATGTCGACCAGGAACAGGTGGTGGCGGC

pEGFP-N1 (Clontech); EGFP-P2-fw/re, template pEYFP-N1 (Clontech). The ends of the products were cut with the appropriate restriction enzymes and cloned into the vector pETDuet-1 as outlined in Figure 4. If required, oligonucleotide linkers, encoding the peptide spacers, were inserted between the subunits. This resulted in the following constructs: pET-mRFP1, pET-t-dimer2(12), pET-ECFP, pET-EGFP, pET-EYFP, pET-mRFP1-12-EGFP, pET-t-dimer2(12)-12-EGFP, pET-EYFP-7-ECFP, pET-mRFP1-12-ECFP, pET-t-dimer2(12)-12-ECFP, pET-mRFP1-12-EYFP, pET-t-dimer2(12)-12-EYFP, where the number between the subunits describes the length and position of the linker (Figure 4).

E. coli Rosetta cells (Novagen) were transformed by these plasmids and grown in 100ml cultures at 37°C until an OD_{600} = 0.7 was reached. A total of 0.5mM IPTG was added, and the cultures were incubated in a shaker at 20°C over night. The cells were harvested by centrifugation for 30min at 3500 x g. One half of the cell pellet was frozen at -80°C for later use. The other half was lysed with 800µl BugBuster reagent (Novagen) following the manufacturer's instructions. The proteins were purified from the clear lysate via their N-terminal His-tags using 300µl HisMag magnetic beads (Novagen) according to the manufacturer's instruction. The spectral properties of the proteins were determined with a Cary Eclipse fluorescence spectrometer (Varian).

The spectral properties and spectral overlap of mRFP1, t-dimer2(12), ECFP, EGFP and EYFP are shown in Figure 5. All spectra were normalised to their maximum excitation and emission (arbitrary value 1). ECFP and EGFP show large spectral overlap but poor distinction between their excitation spectra whereas the mRFP1 excitation has only little overlap with either fluorescence emission (Figure 5a). The well-characterised FRET pair ECFP-EYFP

shows significant overlap between donor emission and acceptor excitation, while the donor and acceptor excitations are sufficiently separated. EYFP also overlaps well with the mRFP1 excitation suggesting that EYFP and mRFP1 are able to form a suitable RET pair (Figure 5b). ECFP and t-dimer2(12) show a surprisingly large spectral overlap despite the large separation of their emission maxima, indicating the potential formation of a suitable RET pair with an emission that is spectrally distinct from the ECFP-EYFP pair (Figure 5c).

Next, RET between the subunits of the fusion proteins was analysed (Figure 6). RET in the mRFP1 fusion proteins indicate an increase in the red fluorescence of 5 times with EGFP compared to mRFP1 on its own (Figure 6a).

EXAMPLE 2 ANALYSIS OF PROTEINACEOUS BRET PAIRS

The use of bioluminescent proteins as DTs requires fluorescent DTs with sufficient spectral overlap to the luminescence emission. The potential for RET between Renilla luciferase (Rluc) as a bioluminescent protein DT and the proteinaceous DTs EGFP, EYFP, t-dimer2(12) and mRFP1 was investigated. Again, as a simple model system for interactions fusion proteins between Rluc and proteinaceous DTs were generated.

The gene for Rluc was amplified via PCR with the following oligos: Rluc-P3-fw/re, template phRL-CMV (Promega). Using appropriate restriction enzymes the PCR product was cloned into the vectors from Example 1 resulting in the constructs pET-Rluc, pET-ECFP-7-Rluc, pET-EGFP-15-Rluc, pET-EYFP-7-Rluc, pET-t-dimer2(12)-15-Rluc and pET-mRFP1-15-Rluc, where the number between the subunits describes the length and position of the linker (Figure 4).

The proteins were expressed in *E. coli* Rosetta cells and

purified as described in Example 1. Luminescence spectra were recorded with a Cary Eclipse (Varian) luminescence spectrometer after the addition of 5 µM coelenterazine h as a substrate for Rluc (Figure 7). The spectra were normalised to their emission maxima (arbitrary value 1).

From the spectra RET ratios (RR) were calculated as a measure for the RET (Figure 8). RET ratios are also an indicator for the affinity and the distance between the DTs. The calculations include a correction term to account for the spectral overlap of the emissions. The ratios were calculated as follows:

EGFP/EYFP:

$$RR^{green/yellow} = \frac{(c_f * green - blue) * c_f * {green}/{blue}}{blue}$$

t-dimer2(12)/mRFP1:

$$RR^{red} = \frac{(c_f * red - blue - green) * c_f * {red}/{(blue + green)}}{blue}$$

where blue = Rluc emission at 475 nm
green = EGFP emission at 510 nm or EYFP emission at 530nm
red = t-dimer2(12) emission at 580 nm or mRFP1 emission at 610 nm
c_f = smallest integer to give positive RR for negative control (Rluc only)

The results show that the t-dimer2(12) emission can be surprisingly well distinguished from either EGFP or EYFP emissions while still achieving a signal separation >80 (EGFP) or >20 (EYFP). EGFP and EYFP as well as t-dimer2(12) and mRFP1 cannot be separated clearly and show significant 'leakage' between the respective channels. Although the mRFP1 emission is separated by 100 nm from

EGFP the signal separation is only about 10 because Rluc cannot substantially activate mRFP1 and as a consequence only a weak red fluorescence signal is detected.

EXAMPLE 3 ANALYSIS OF NON-PROTEINACEOUS BRET PAIRS

The choice of DTs is not restricted to proteinaceous molecules. Small fluorescent molecules as DTs may for many applications offer advantages as they are available with a wider range of spectral properties, and their smaller size makes them less likely to interfere with the function of the attached interacting group. As a model system for biological interactions the strong affinity of the protein streptavidin to the biotin-group was used. Streptavidins are available as conjugates with many different small molecule fluorescence dyes.

The *E. coli* enzyme biotin ligase (birA) mediates in the presence of ATP the attachment of a biotin-group to a lysine-residue of a specific 13-aminoacid peptide sequence called 'avitag' (Schatz, P.J. (1993) Bio/Technology 11, 1138-1143). The coding sequence of the avitag was inserted into the construct pET-Rluc as a linker consisting of the hybridised oligonucleotides AvitagN-fw/re (Table 3). The linker was cloned into position 1 (Figure 4) of this construct. The coding sequence for biotin ligase was amplified with the oligonucleotides birA-fw/re using *E. coli* Top10 as a template. The birA gene was cloned into position 4 of the construct (Figure 4) to co-express biotin ligase for a quantitative biotinylation of the target sequence. The resulting construct for the expression of a biotinylated Rluc protein was called pET-Avi-15-Rluc/birA.

The protein was expressed in *E. coli* Rosetta cells and purified as described in Example 1. To the biotin-Rluc

protein solution BSA was added to a final concentration of 2 mg/ml to prevent an unspecific interaction of the proteins. An approximately equimolar amount of streptavidin conjugates was added to the solution and luminescence spectra were recorded with a Cary Eclipse (Varian) luminescence spectrometer after the addition of 5 µM coelenterazine h as a substrate for Rluc (Figure 9). The spectra were normalised to their emission maxima (arbitrary value 1).

All small fluorescence dyes were suitable as DTs and resulted in RET signals in the presence of the substrate coelenterazine h (Figure 9). Alexa fluor 488 had the biggest spectral overlap with the Rluc emission and thus showed the highest RET signal. Surprisingly however, Alexa Fluor 594, the spectrally most distant dye used, resulted in a RET signal of similar or greater magnitude than the spectrally more overlapping dyes Alexa Fluor 555 and Alexa Fluor 568. Non-biotinylated Rluc was used as a negative control and did not yield a RET signal, demonstrating the specificity of the biotin-streptavidin model interaction.

An important aspect of assays according to this invention is that they provide a quantitative measure for biological interactions. Serial dilution of streptavidin conjugates were incubated with biotinylated Rluc plus 2 mg/ml BSA. After the addition of 5 µM coelenterazine h luminescence spectra were recorded, and the RET ratios were calculated using the equations from Example 2 with substitution of the emission maxima for the respective dyes. Figure 10 shows that for both conjugates, Oregon green and Alexa Fluor 594, the RET ratio is proportional to the common logarithm of the streptavidin concentration, demonstrating that the assays provide a quantitative measure for interactions. It is also remarkable that Alexa Fluor 594, despite its little spectral overlap with the Rluc emission and thus comparably small signal, is as sensitive as

Oregon green in detecting the biotin-streptavidin interaction.

EXAMPLE 4 SIMULTANEOUS, MULTIPLEX RET DETECTION

According to this invention multiple biological interactions can be detected simultaneously and in a quantitative manner ('multiplexing'). To explore this possibility the model systems used in Examples 2 and 3 were adapted for multiplexing.

In Example 2, the proteinaceous DT pair EGFP-t-dimer2(12) was identified as potential multiplex combination due to the sufficient spectral resolution between their emission maxima. The concentrations of the EGFP-15-Rluc and t-dimer2(12)-15-Rluc fusion proteins were adjusted to similar concentrations. The protein solutions were mixed stepwise with ratios of 5:0, 4:1…1:4, 0:5. The Rluc substrate coelenterazine h was added to a final concentration of 5µM and luminescence spectra were recorded. RET ratios were calculated and normalised to the highest value (arbitrary value 1) for an easier comparison of the two channels (Figure 11).

Multiplex detection was further tested using the biotin-streptavidin interaction model. Biotinylated Rluc plus 2 mg/ml BSA was mixed with an equimolar amount of streptavidin-Oregon green in one tube and with streptavidin-Alexa Fluor 594 in a separate tube. The solutions were mixed stepwise with ratios of 5:0, 4:1…1:4, 0:5. The Rluc substrate coelenterazine h was added to a final concentration of 5µM and luminescence spectra were recorded. RET ratios were calculated and again normalised to the highest value (arbitrary value 1) for an easier comparison of the two channels (Figure 11).

In summary, this example demonstrates that this invention

enables multiplex detection of biological interactions in a variety of applications and embodiments. This has been shown to be independent of the model used and independent of the nature of the DT.

EXAMPLE 5 RET IN COMPLEX MOLECULAR ASSOCIATES

The analysis of complex molecular associates containing more than two components requires a detection system that yields distinct signals for possible combinations of components within the associate. As a model for the interactions within an associate of three components a fusion protein of three DTs was analysed.

The coding sequences for EGFP and ECFP or Rluc were cloned together with an oligonucleotide linker into the construct pET-mRFP1 resulting in the constructs pET-mRFP1-12-EGFP-ECFP and pET-mRFP1-12-EGFP-Rluc (Figure 4). The proteins were expressed in *E. coli* Rosetta cells and purified as described in Example 1.

The spectral properties of the mRFP1-12-EGFP-ECFP fusion construct were determined and compared to mRFP1 and the mRFP1-12-EGFP fusion protein (Figure 12). The spectra were normalised to the emission of mRFP1 when excited at 560nm to correct for differences in concentration and levels of maturation of the red fluorophore. Activation of ECFP results in the activation EGFP as well as mRFP1. The presence of EGFP markedly increased the fluorescence emission of mRFP1. The effect was maximal at excitation wavelengths between 420 and 430nm, the excitation maximum of ECFP.

A further activation of EGFP also resulted in the activation of mRFP1 providing additional evidence for the association of these two proteins. Activation of EGFP by ECFP indicated the association of these proteins and the

activation of mRFP1 by ECFP demonstrated that all three proteins were part of the same associate (Table 1 'AND').

The luminescence spectrum of mRFP1-12-EGFP-Rluc was recorded after the addition of 5 µM coelenterazine h (Figure 13). Rluc activates EGFP, which in turn activates mRFP1 resulting in an activation of the green fluorescence at 510 nm as well as an activation of the red fluorescence at 610 nm. The spectrum in Figure 13a also shows an increase of the green fluorescence of the mRFP1-12-EGFP-Rluc construct compared to the mRFP1-15-EGFP construct. This is most likely because the maturation of the red fluorophore of mRFP1 in this particular construct is slower and a significantly larger proportion remains at the green fluorescent intermediate stage than in unfused mRFP1 or mRFP1 fused to only one additional subunit. Compared to the mRFP1-15-EGFP construct only about 50% of the fluorophore is formed. Even despite the lower abundance of the red fluorophore, the mRFP1 fluorescence emission is significantly higher compared to the mRFP1-15-Rluc construct, which does not include a EGFP subunit.

Additionally, the activation of EGFP results in an activation of mRFP1 indicating the association between EGFP and mRFP1. The activation of EGFP by Rluc indicates an association between these two proteins and the additional activation of mRFP1 by Rluc indicates that all three proteins are part of the same associate (Table 1 'AND').

Taken together, these result demonstrates that RET signals combine to yield information on the presence of three components within an associate.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A multi-component detection system comprising:

i). a first agent comprising a first interacting group coupled directly or indirectly to a first detection tag, which tag emits light of a first wavelength upon activation by a substrate or excitation light which produces a first activated detection tag;

ii). a second agent comprising a second interacting group coupled directly or indirectly to a second detection tag, which tag can accept the energy from the first detection tag in i) when the first and second interacting groups are associated and an appropriate substrate or excitation light for the first detection tag in i) is present thereby producing a second activated detection tag that emits light of a second wavelength;

iii). a third agent comprising a third interacting group coupled directly or indirectly to a third detection tag that can accept the energy from the second activated detection tag in ii) when the second and third interacting groups are associated and an appropriate excitation light for the second detection tag in ii) is present and that can accept the energy from the second activated detection tag in ii) when the first, second and third interacting groups are associated and an appropriate substrate or excitation light for the first detection tag in i) is present to produce a third activated detection tag that emits light of a third wavelength, but said third detection tag is not substantially activated by the first activated detection tag in i) when the first and third interacting groups are associated;

iv). an appropriate substrate or excitation light source to activate the detection tags in i) and ii); and

v). a means of detecting said emitted light.

2. A multi-component detection system comprising:

i). a first agent comprising a first interacting group coupled directly or indirectly to a first detection tag, which tag emits light of a first wavelength upon activation by a substrate or excitation light which produces a first activated detection tag;

ii). a second agent comprising a second interacting group coupled directly or indirectly to a second detection tag, which tag can accept the energy from the first detection tag in i) when the first and second interacting groups are associated and an appropriate substrate or excitation light for the first detection tag in i) is present thereby producing a second activated detection tag that emits light of a second wavelength;

iii). a third agent comprising a third interacting group coupled directly or indirectly to a third detection tag consisting of a non-fluorescent quencher molecule that can accept the energy from:

a). the first activated detection tag when the first and third interacting groups are associated; and/or

b). the second activated detection tag when the second and third interacting groups are associated and an appropriate substrate or excitation light for the first and/or second detection tag is present, whereby the light emission from the first and/or second activated detection tag is decreased;

iv). an appropriate substrate or excitation light source to activate the detection tags in i) and ii); and

v). a means of detecting said emitted light.

3. A multi-component detection system comprising:

i). a first agent comprising a first interacting group coupled directly or indirectly to a first detection tag, which tag emits light of a first wavelength upon activation by a substrate or excitation light which produces a first activated detection tag;

ii). a second agent comprising a second

interacting group coupled directly or indirectly to a second detection tag, which tag emits light of a second wavelength upon activation by a substrate or excitation light, which produces a second activated detection tag;

iii). a third agent comprising a third interacting group coupled directly or indirectly to a third detection tag, which tag can accept the energy from the first activated detection tag when the first and third interacting groups are associated and an appropriate substrate or excitation light for the first detection tag is present to produce a third activated detection tag that emits light of a third wavelength;

iv). a fourth agent comprising a fourth interacting group coupled directly or indirectly to a fourth detection tag, which tag can accept the energy from the second activated detection tag when the second and fourth interacting groups are associated and an appropriate substrate or excitation light for the second detection tag is present to produce a fourth activated detection tag that emits light of a fourth wavelength;

v). an appropriate substrate or excitation light source to activate the first and second detection tags, and

vi). a means of detecting said emitted light.

4. A multi-component detection system comprising:

i). a first agent comprising a first interacting group coupled directly or indirectly to a first detection tag, which tag emits light of a first wavelength upon activation by a substrate or excitation light, which produces a first activated detection tag;

ii). a second agent comprising a second interacting group coupled directly or indirectly to a second detection tag, which tag can accept the energy from the first detection tag when the first and second interacting groups are associated and an appropriate substrate or excitation light for the first detection tag

is present thereby producing a second activated detection tag that emits light of a second wavelength;

iii). a third agent comprising a third interacting group coupled directly or indirectly to a third detection tag that can accept the energy from the first activated detection tag when the first and third interacting groups are associated and an appropriate substrate or excitation light for the first detection tag is present to produce a third activated detection tag that emits light of a third wavelength;

iv). an appropriate substrate or excitation light source to activate the first detection tag, and

v). a means of detecting said emitted light.

5. A system according to any one of claims 1 to 4, wherein the interacting group is selected from the group consisting a compounds, proteins, protein domains, protein loops, protein termini, peptides, hormones, protein-lipid complexes, lipids, carbohydrates, carbohydrate-containing compounds, nucleic acids, oligonucleotides, pharmaceutical agents, pharmaceutical drug targets, antibodies, antigenic substances, viruses, bacteria, and cells.

6. A system according to any one of claims 1 to 4, wherein the interacting group is selected from the group consisting of carbohydrates, proteins, drugs, chromophores, antigens, chelating compounds, molecular recognition complexes and combinations thereof.

7. A system according to claim 5, wherein the nucleic acid molecule comprises DNA, RNA including heterogeneous ribonucleic acid (hnRNA), transfer ribonucleic acid (tRNA), messenger ribonucleic acid (mRNA), or ribosomal ribonucleic acid (rRNA).

8. A system according to any one of claims 1 to 7, wherein external stimuli are applied to directly or

indirectly modulate the association of interacting groups.

9. A system according to claim 8 wherein the external stimuli are reagents comprising organic and inorganic molecules, proteins, nucleic acids, carbohydrates, lipids, ligands, drug compounds, agonists, antagonists, inverse agonists or compounds.

10. A system according to claim 8 wherein the external stimuli are changes of conditions including temperature, ionic strength or pH.

11. A system according to any one of claims 1 to 10, wherein the detection tag is selected from the group consisting of a bioluminescent protein, a fluorescent protein, a fluorescent moiety or a non-fluorescent quencher.

12. A system according to claim 11, wherein the bioluminescent protein is selected from the group consisting of luciferase, galactosidase, lactamase, peroxidase or any protein capable of luminescence in the presence of a suitable substrate.

13. A system according to claim 11, wherein the fluorescent protein selected from the group consisting of green fluorescent protein (GFP) or variants thereof, blue fluorescent variant of GFP (BFP), cyan fluorescent variant of GFP (CFP), yellow fluorescent variant of GFP (YFP), enhanced GFP (EGFP), enhanced CFP (ECFP), enhanced YFP (EYFP), GFPS65T, Emerald, Topaz, GFPuv, destabilised EGFP (dEGFP), destabilised ECFP (dECFP), destabilised EYFP (dEYFP), HcRed, t-HcRed, DsRed, DsRed2, t-dimer2, t-dimer2(12), mRFP1, pocilloporin, Renilla GFP, Monster GFP, paGFP, Kaede protein and kindling protein, Phycobiliproteins and Phycobiliprotein conjugates including B-Phycoerythrin, R-Phycoerythrin and

Allophycocyanin or any other protein capable of fluorescence.

14. A system according to claim 11, wherein the fluorescent moiety is selected from the group consisting of Alexa Fluor dyes and derivatives, Bodipy dyes and derivatives, Cy dyes and derivatives, fluorescein and derivatives, dansyl, umbelliferone, fluorescent and luminescent microspheres, fluorescent nanocrystals, Marina Blue, Cascade Blue, Cascade Yellow, Pacific Blue, Oregon Green and derivatives, Tetramethylrhodamine and derivatives, Rhodamine and derivatives, Texas Red and derivatives, rare earth element chelates or any combination or derivative thereof or any other molecule with fluorescent properties.

15. A system according to claim 11 wherein the non-fluorescent quencher is selected from the group consisting of dabcyl, non-fluorescent pocilloporins, QSY-7, QSY-9, QSY-21, QSY-35, BHQ-1, BHQ-2, BHQ-3 or any known non-fluorescent chromophore with the ability to absorb light and to quench fluorescence and/or luminescence.

16. A system according to any one of claims 1 to 15, wherein the interacting group and detection tag are coded for in a fusion protein construct.

17. A recombinant DNA encoding a fusion protein construct according to claim 16.

18. A fusion gene that comprises a recombinant DNA according to claim 17.

19. A DNA cassette comprising a promoter operably linked to one or more fusion protein genes according to claims 16-18.

20. A vector comprising a fusion gene according to claim 19.

21. A host cell transformed or transfected by a vector according to claim 20.

22. A host cell according to claim 21, wherein the cell is human, mammalian, insect, plant, bacterial, or yeast.

23. A vector according to claim 20, wherein the gene construct is under the control of a constitutive promoter.

24. A vector according to claim 23, wherein the constitutive promoter is selected from the group consisting of CMV, SV40, RSV, EF-1 a, Tk, and AdML, when the cell to be transformed or transfected is mammalian.

25. A vector according to claim 23, wherein the constitutive promoter is selected from the group consisting of T7, AraBAD, trc, pL, tac, and lac, when the cell to be transformed or transfected is a bacterial cell.

26. A vector according to claim 23, wherein the constitutive promoter is selected from the group consisting of nmt1, gal1, gal10, TEF1, AOX1, GAP, and ADH1, when the cell to be transformed or transfected is a yeast cell.

27. A virus comprising a fusion gene according to claim 19.

28. A host cell infected by a viral vector according to claim 27.

Dated this 19th day of November 2003

The University of Western Australia

By their Patent Attorneys
GRIFFITH HACK

Fellows Institute of Patent and
Trade Mark Attorneys of Australia

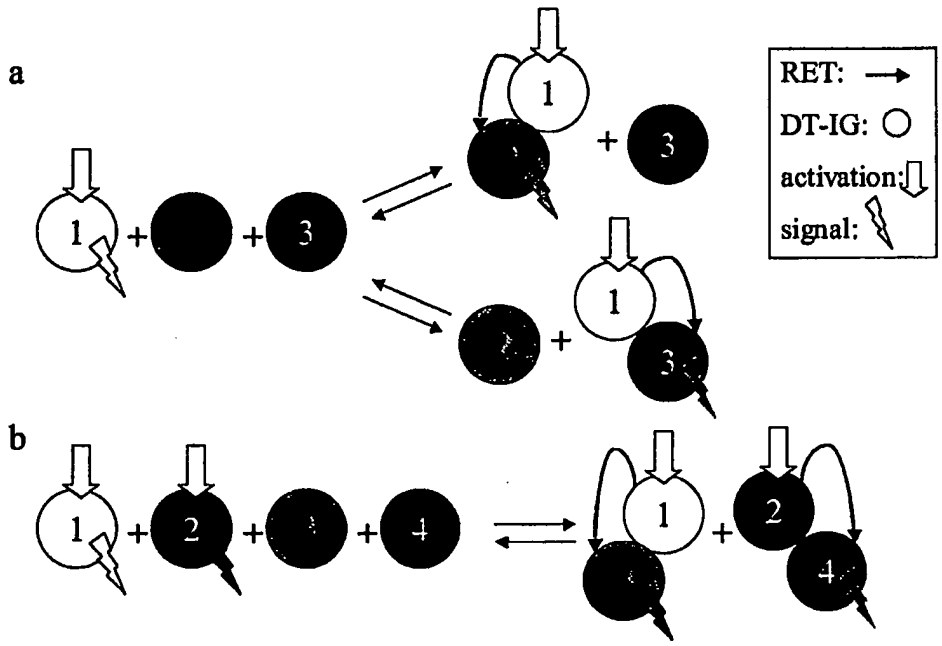


FIGURE 1

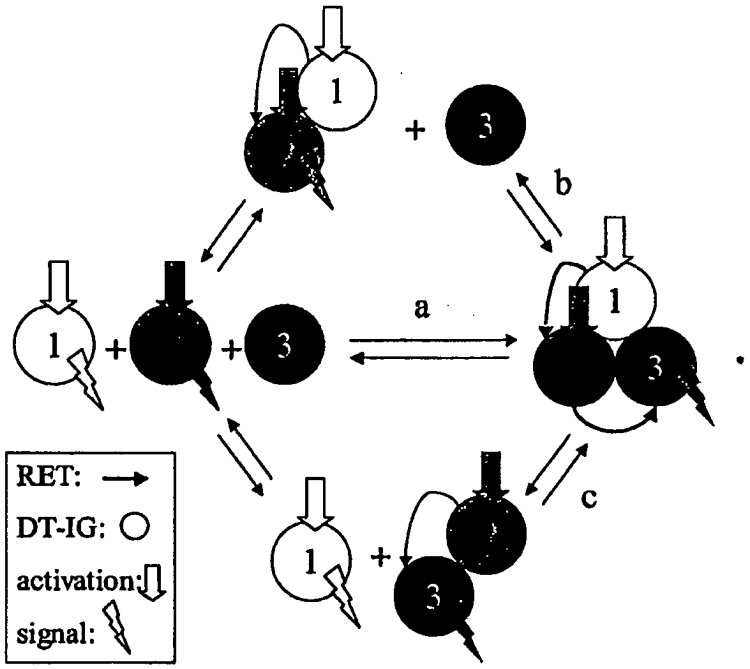


FIGURE 2

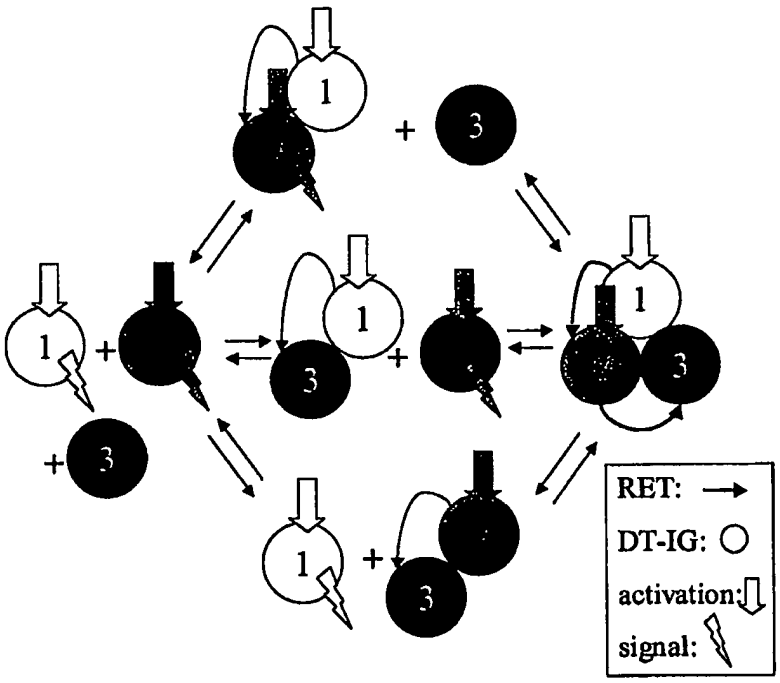


FIGURE 3

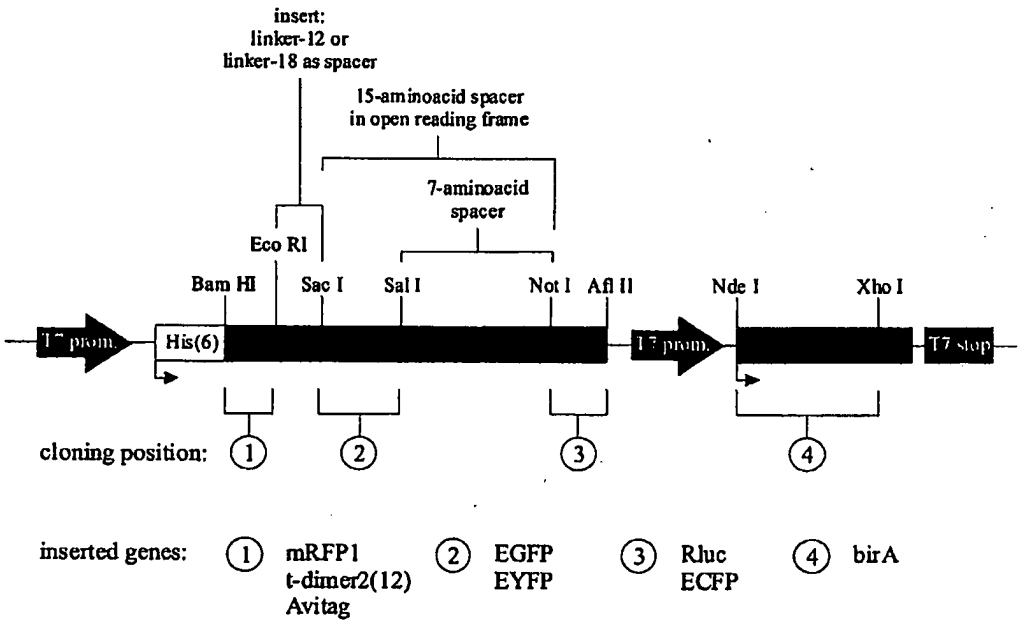
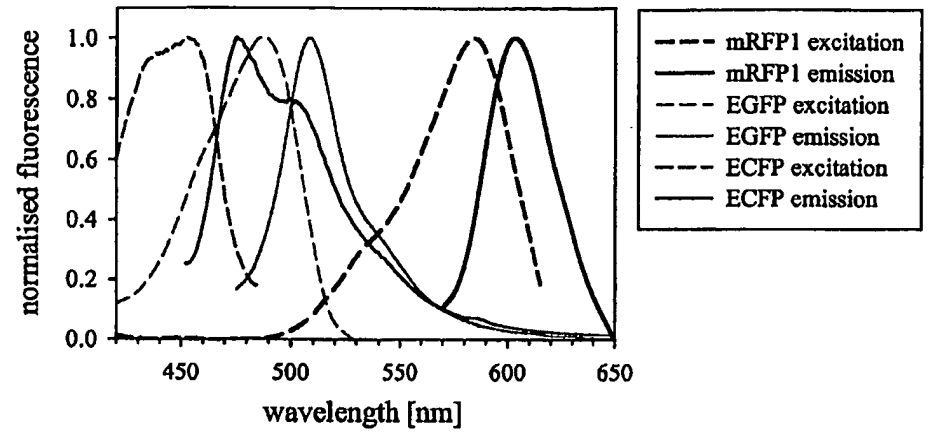
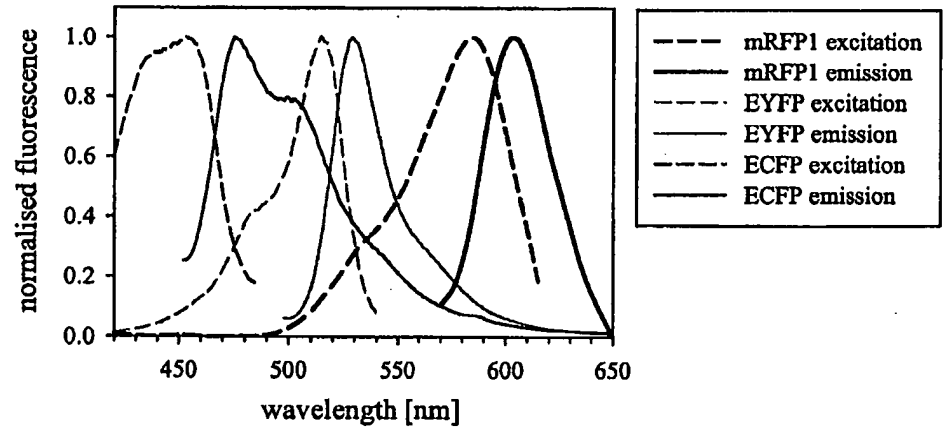

insert:
linker-12 or
linker-18 as spacer
15-aminoacid spacer
in open reading frame
7-aminoacid
spacer
Eco RI
Bam HI
Sac I
Sal I
Not I
Afl II
Nde I
Xho I
T7 prom.
His(6)
T7 prom.
T7 stop
cloning position:
1
2
3
4
inserted genes:
1 mRFP1
t-dimer2(12)
Avitag
2 EGFP
EYFP
3 Rluc
ECFP
4 birA

FIGURE 4

a


normalised fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
450
500
550
600
650
wavelength [nm]
mRFP1 excitation
mRFP1 emission
EGFP excitation
EGFP emission
ECFP excitation
ECFP emission

b


normalised fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
450
500
550
600
650
wavelength [nm]
mRFP1 excitation
mRFP1 emission
EYFP excitation
EYFP emission
ECFP excitation
ECFP emission

c

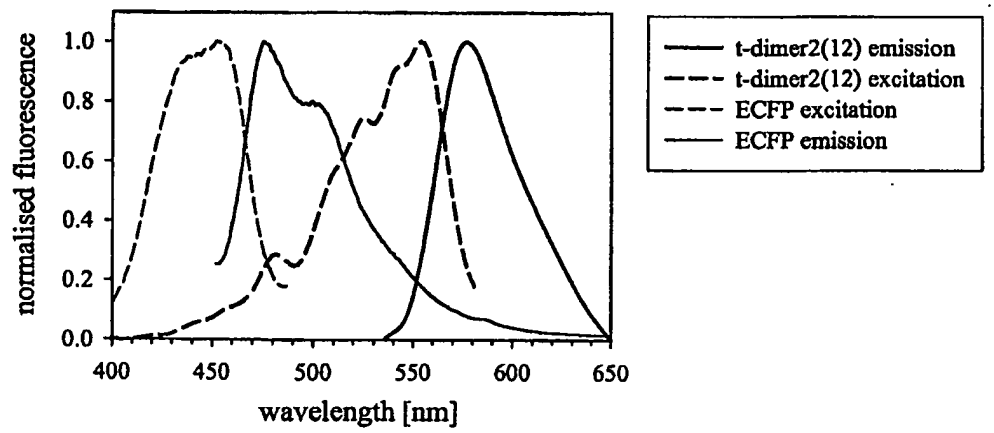

normalised fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
400
450
500
550
600
650
wavelength [nm]
t-dimer2(12) emission
t-dimer2(12) excitation
ECFP excitation
ECFP emission

FIGURE 5

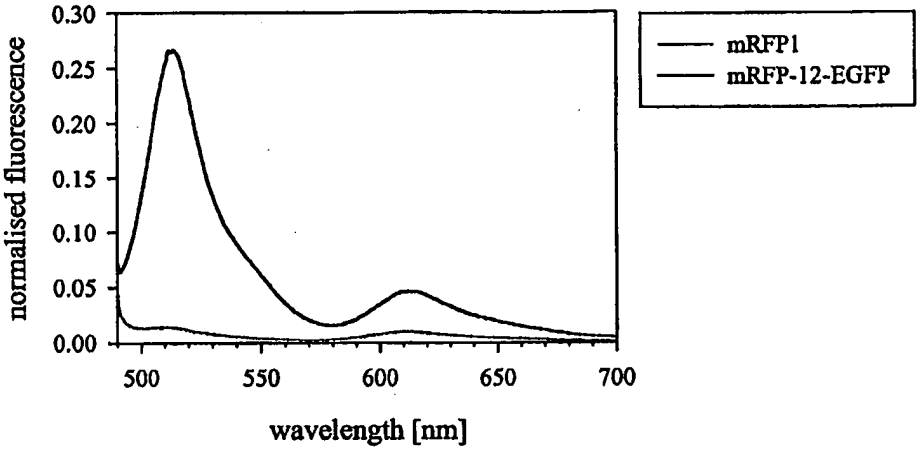

0.30
0.25
0.20
0.15
0.10
0.05
0.00
normalised fluorescence
500
550
600
650
700
wavelength [nm]
mRFP1
mRFP-12-EGFP

FIGURE 6

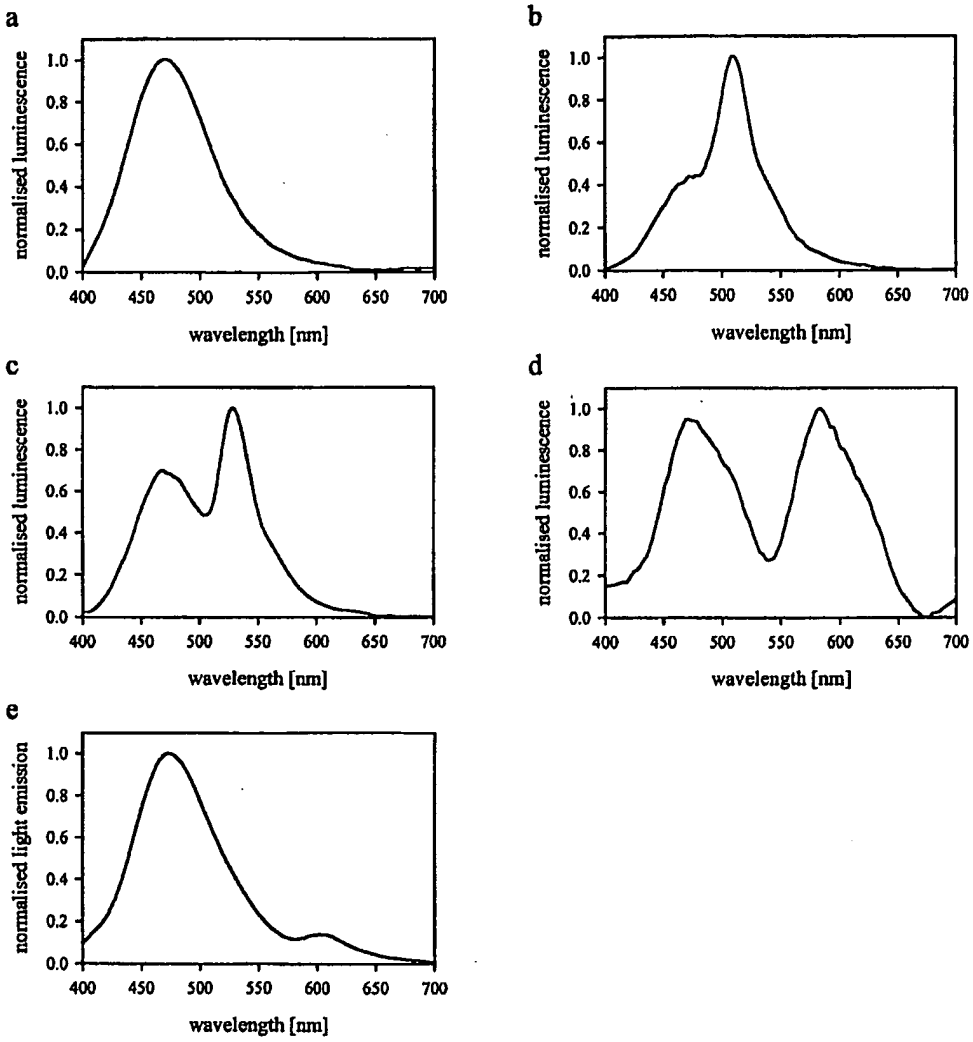

a
normalised luminescence
1.0
0.8
0.6
0.4
0.2
0.0
400 450 500 550 600 650 700
wavelength [nm]
b
normalised luminescence
1.0
0.8
0.6
0.4
0.2
0.0
400 450 500 550 600 650 700
wavelength [nm]
c
normalised luminescence
1.0
0.8
0.6
0.4
0.2
0.0
400 450 500 550 600 650 700
wavelength [nm]
d
normalised luminescence
1.0
0.8
0.6
0.4
0.2
0.0
400 450 500 550 600 650 700
wavelength [nm]
e
normalised light emission
1.0
0.8
0.6
0.4
0.2
0.0
400 450 500 550 600 650 700
wavelength [nm]

FIGURE 7

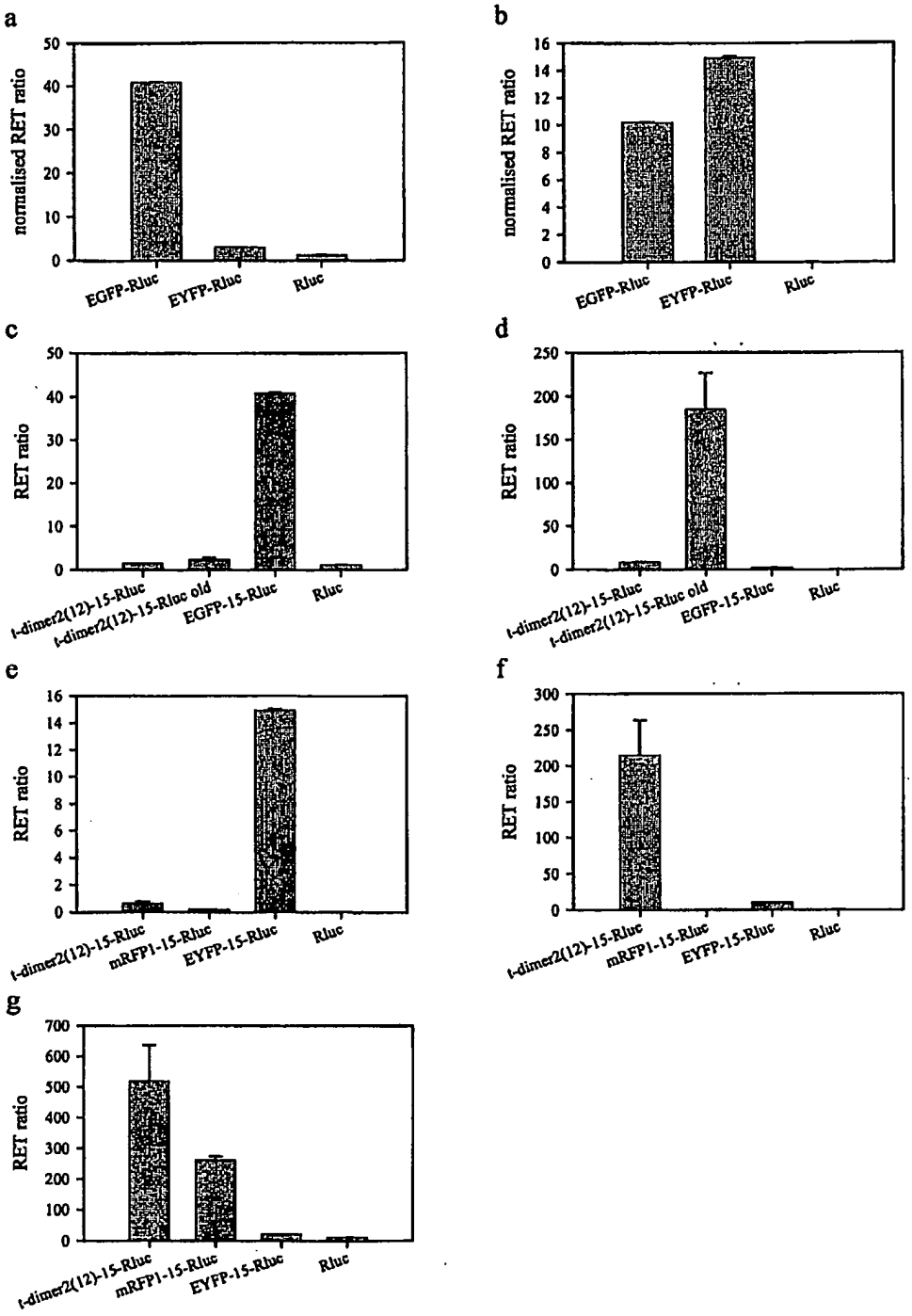

a
normalised RET ratio
50
40
30
20
10
0
EGFP-Rluc
EYFP-Rluc
Rluc
b
normalised RET ratio
16
14
12
10
8
6
4
2
0
EGFP-Rluc
EYFP-Rluc
Rluc
c
RET ratio
50
40
30
20
10
0
t-dimer2(12)-15-Rluc
t-dimer2(12)-15-Rluc old
EGFP-15-Rluc
Rluc
d
RET ratio
250
200
150
100
50
0
t-dimer2(12)-15-Rluc
t-dimer2(12)-15-Rluc old
EGFP-15-Rluc
Rluc
e
RET ratio
16
14
12
10
8
6
4
2
0
t-dimer2(12)-15-Rluc
mRFP1-15-Rluc
EYFP-15-Rluc
Rluc
f
RET ratio
300
250
200
150
100
50
0
t-dimer2(12)-15-Rluc
mRFP1-15-Rluc
EYFP-15-Rluc
Rluc
g
RET ratio
700
600
500
400
300
200
100
0
t-dimer2(12)-15-Rluc
mRFP1-15-Rluc
EYFP-15-Rluc
Rluc

FIGURE 8

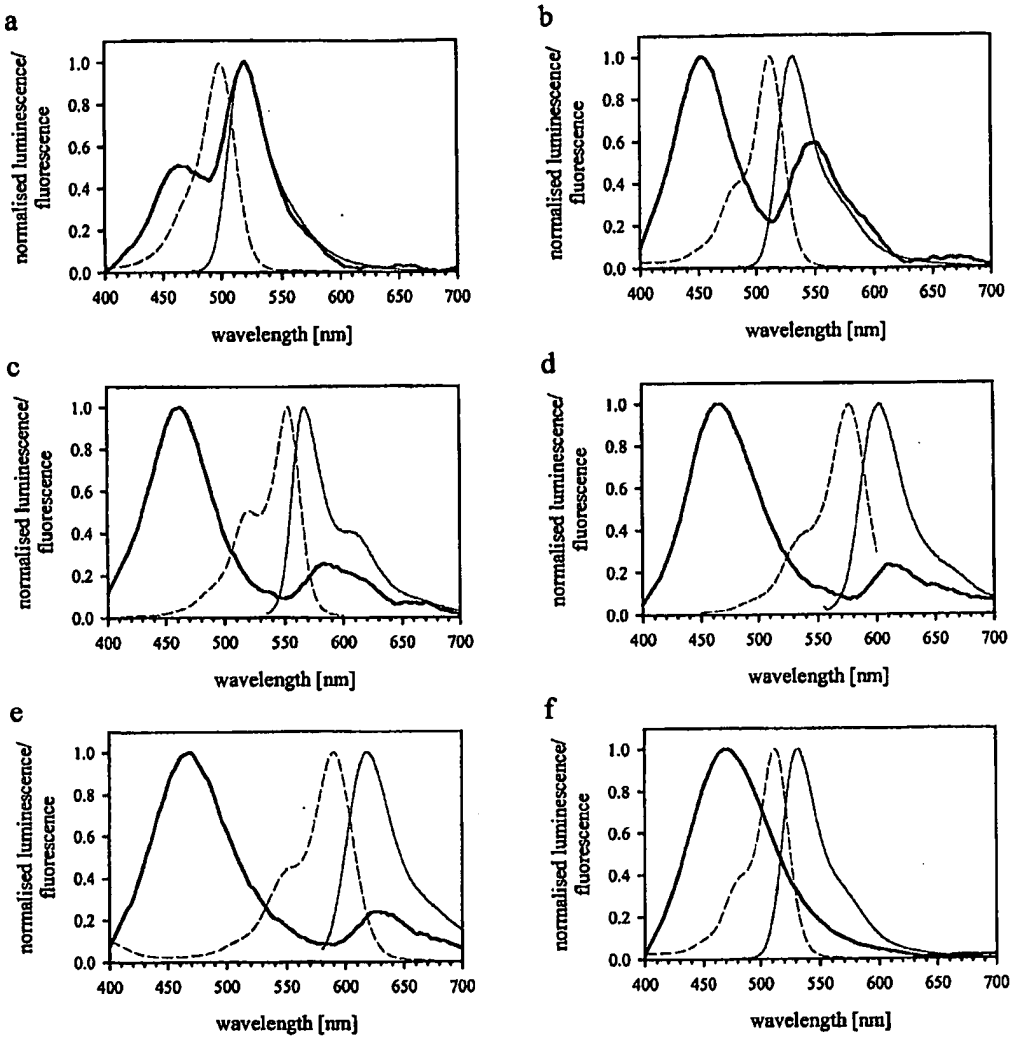

a
b
c
d
e
f
normalised luminescence/ fluorescence
wavelength [nm]
0.0
0.2
0.4
0.6
0.8
1.0
400
450
500
550
600
650
700

FIGURE 9

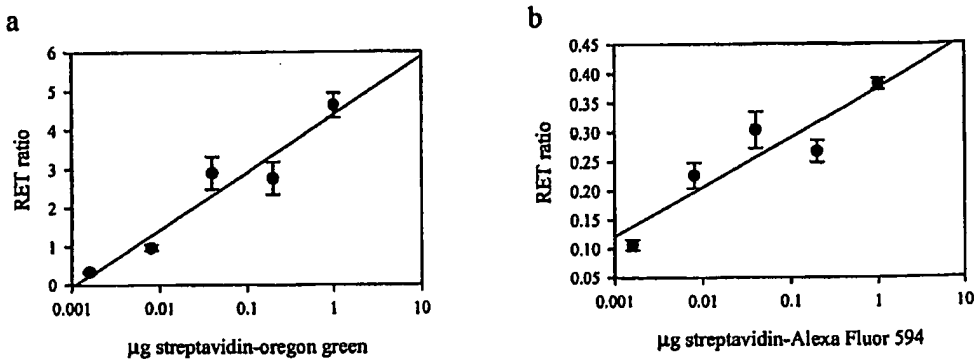
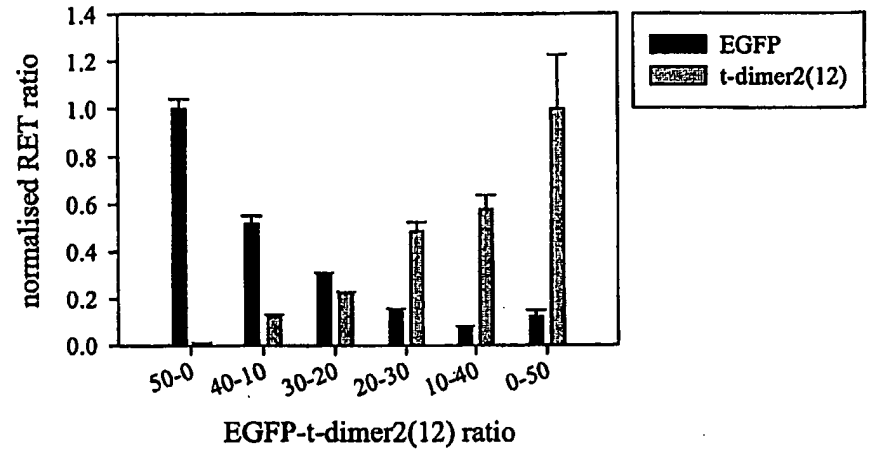

a
RET ratio
0
1
2
3
4
5
6
0.001
0.01
0.1
1
10
µg streptavidin-oregon green
b
RET ratio
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.001
0.01
0.1
1
10
µg streptavidin-Alexa Fluor 594

FIGURE 10

a


normalised RET ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
EGFP
t-dimer2(12)
50-0
40-10
30-20
20-30
10-40
0-50
EGFP-t-dimer2(12) ratio

b

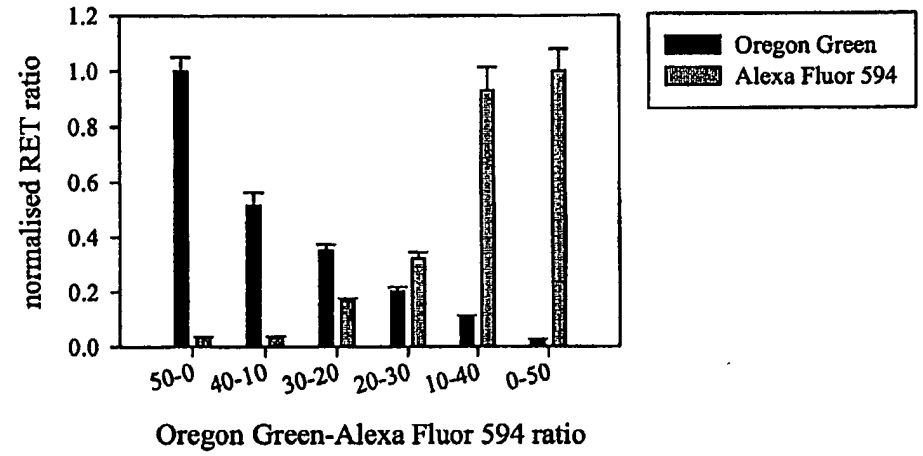

normalised RET ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Oregon Green
Alexa Fluor 594
50-0
40-10
30-20
20-30
10-40
0-50
Oregon Green-Alexa Fluor 594 ratio

FIGURE 11

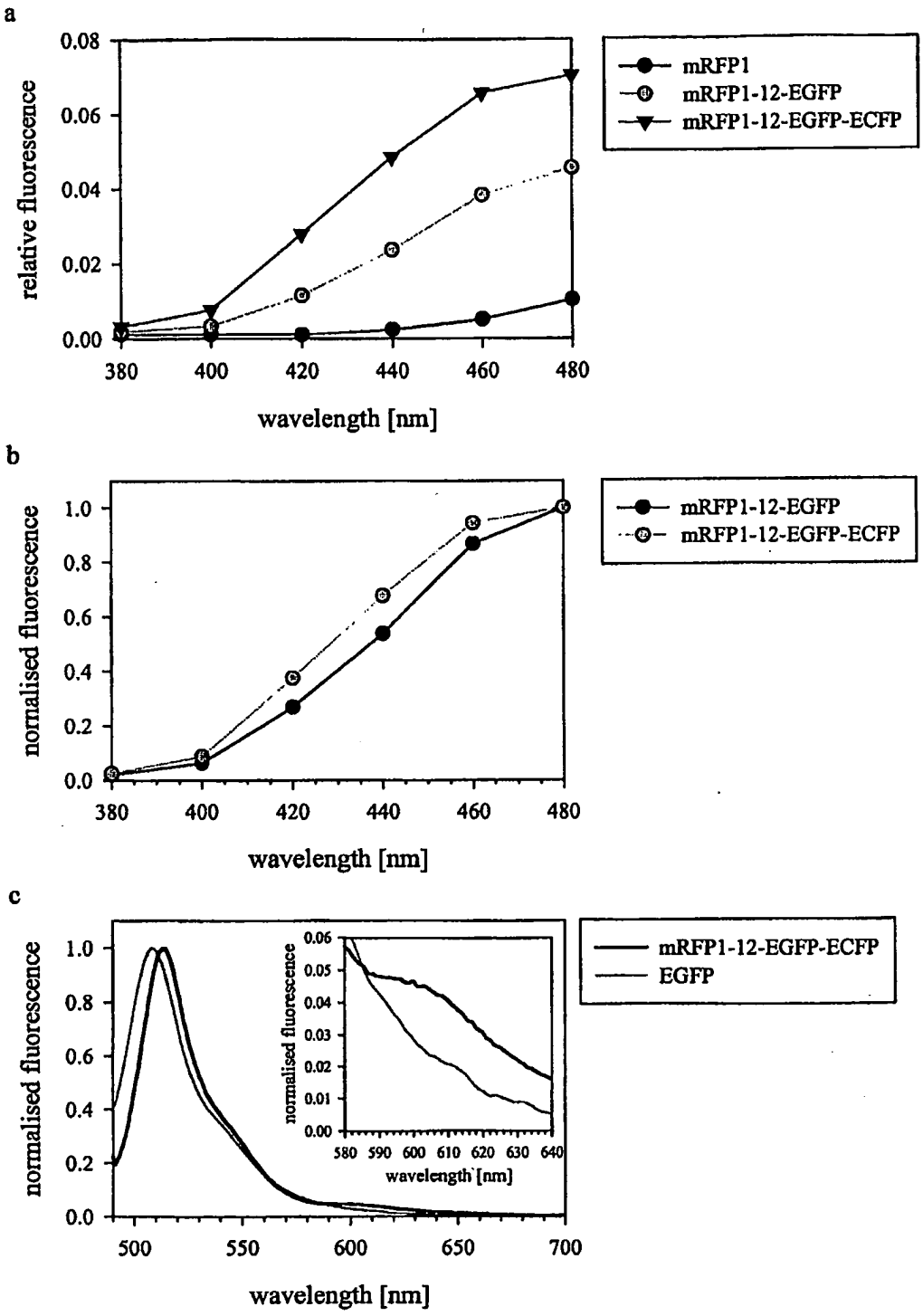
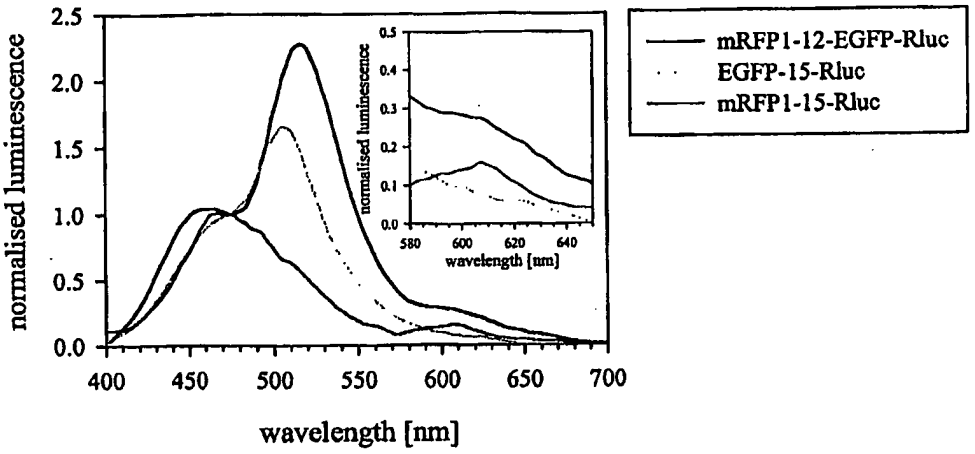

a
relative fluorescence
0.08
0.06
0.04
0.02
0.00
380
400
420
440
460
480
wavelength [nm]
mRFP1
mRFP1-12-EGFP
mRFP1-12-EGFP-ECFP
b
normalised fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
380
400
420
440
460
480
wavelength [nm]
mRFP1-12-EGFP
mRFP1-12-EGFP-ECFP
c
normalised fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
500
550
600
650
700
wavelength [nm]
normalised fluorescence
0.06
0.05
0.04
0.03
0.02
0.01
0.00
580
590
600
610
620
630
640
wavelength [nm]
mRFP1-12-EGFP-ECFP
EGFP

FIGURE 12

a


normalised luminescence
wavelength [nm]
mRFP1-12-EGFP-Rluc
EGFP-15-Rluc
mRFP1-15-Rluc

b

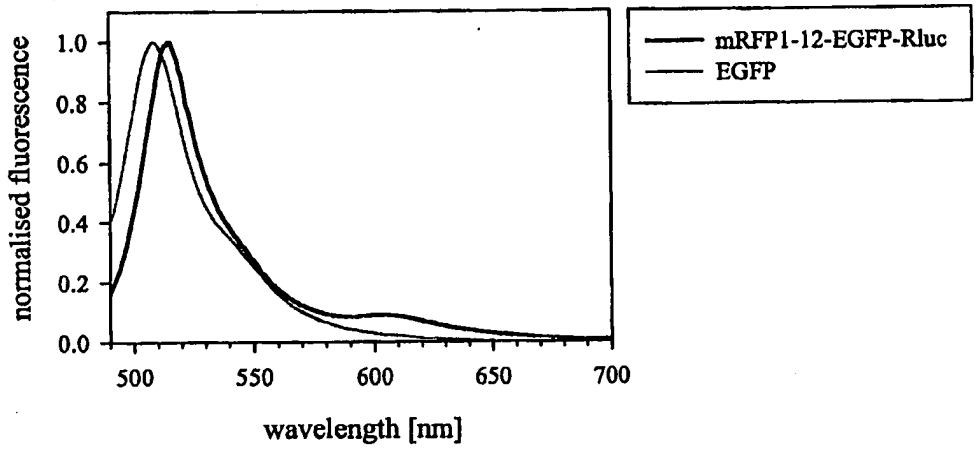

normalised fluorescence
wavelength [nm]
mRFP1-12-EGFP-Rluc
EGFP

FIGURE 13